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13. Abstract *(Maximum 200 Words)* *(abstract should contain no proprietary or confidential information)*

We have proposed to develop novel antibody fusion proteins in which human IgG3 specific for the breast tumor associated antigen HER2/*neu* will be genetically fused to the cytokines IL-2, IL-12, and GM-CSF. The anti-HER2/*neu* antibody-cytokine fusion proteins were intended to localize the cytokine at the tumor where it is expected to elicit an immune response. To accomplish our goals we proposed three specific aims. Specific Aim 1: To produce IL-2, IL-12, GM-CSF antibody fusion proteins specific for HER2/*neu*. Specific Aim 2: To evaluate the properties of the antibody fusion proteins *in vitro*. Specific Aim 3: To determine the properties of the antibody fusion proteins *in vivo* and their effectiveness in causing anti-tumor response. These there specific aims have been accomplished by the end of the third year of the U.S. Army Award. Treatment with these antibody fusion proteins resulted in significant anti-tumor activity in murine tumor models expressing human HER2/*neu* under conditions in which the anti-HER2/*neu* antibody alone failed to confer protection. In addition, have also found that anti-HER2/*neu* antibody fusion proteins are effective enhancers of extracellular domain HER2/*neu* protein vaccination. Our results suggest that the three antibody fusion proteins we have developed: anti-HER2/*neu* IgG3-(GM-CSF), anti-HER2/*neu* IgG3-(IL2), and anti-HER2/*neu* IgG3-(IL12) may be effective prophylactic and therapeutic agents against HER2/*neu* expressing tumors in human.

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INTRODUCTION

In this grant we proposed to explore the use of genetically engineered antibodies as therapeutic agents specifically attempting to augment and potentiate the host immune defense systems against breast cancer. The antibodies were to be specific for HER2/*neu*, a molecule present on the surface of many breast cancers; its increased expression is associated with poor prognosis. To these antibodies we proposed to join the cytokines IL-2, IL-12, and GM-CSF. Expression of these cytokines by cancer cells has been shown to render them immunogenic. The anti-HER2/*neu* antibody fusion proteins were intended to localize the cytokine at the tumor where it is expected to elicit an immune response. To accomplish our goals we proposed three specific aims. Specific Aim 1: To produce IL-2, IL-12, GM-CSF antibody fusion proteins specific for HER2/*neu*. Specific Aim 2: To evaluate the properties of the antibody fusion proteins *in vitro*. Specific Aim 3: To determine the properties of the antibody fusion proteins *in vivo* and their effectiveness in causing anti-tumor response. These there specific aims have been accomplished by the end of the third year of the present award.

BODY

Prior to the funding of this proposal we had already completed the initial characterization of an anti-HER2/*neu* IgG3-(IL-12) fusion protein (1). The publication describing these initial studies is attached as an appendix. Studies to define the mechanism of action of anti-HER2/*neu* IgG3-(IL-12) were conducted during the present funding. We found that the anti-tumor activity of antibody-(IL-12) is dose-dependent and comparable or better than recombinant IL-12 using subcutaneous and metastatic models of disease. The anti-tumor activity of anti-HER2/*neu* IgG3-(IL-12) is reduced in Rag2 knockout mice, suggesting that T cells play a role in tumor rejection. In SCID-beige mice, the anti-tumor activity is further reduced, suggesting that NK cells and/or macrophages are also important. The isotype of the antibody response to HER2/*neu* is consistent with a switch from a Th2 to a Th1 immune response and infiltration of mononuclear cells is seen in tumors from mice treated with anti-HER2/*neu* IgG3-(IL-12). Immunohistochemistry reveals that anti-HER2/*neu* IgG3-(IL-12) is anti-angiogenic. Thus, the mechanism of the anti-tumor activity exhibited by anti-HER2/*neu* IgG3-(IL-12) is highly complex and involves a combination of T and NK cell activity, a switch to a Th1 immune response and anti-antiogenic activity. This was the first study comparing the *in vivo* anti-tumor activity of an antibody-(IL-12) fusion protein and free IL-12. Our results are described in a paper entitled "Mechanism of antitumor activity of a single-chain IL-12 IgG3 antibody fusion protein (mscIL-12.her2.IgG3)" by Peng L, Penichet M.L., Dela Cruz J.S., Sampogna S.L., and Morrison S.L. which was published by Journal of Interferon and Cytokine Research (2) (see appendix).

During the time of this award we completed the construction, expression and characterization (*in vitro* and *in vivo*) of an anti-HER2/*neu* IgG3-(GM-CSF) fusion protein (3). Anti-HER2/*neu* IgG3-(GM-CSF) expressed in myeloma cells was correctly assembled and secreted. Anti-HER2/*neu* IgG3-(GM-CSF) binds HER2/*neu* expressing cells with an affinity similar to that of the parental antibody. The GM-CSF in the fusion protein was able to support the growth of a GM-CSF dependent murine myeloid cell line, FDC-P1. The antibody fusion protein was comparable to the parental antibody in its ability to effect ADCC mediated tumor cell lysis and activate J774.2 macrophage cells so that they could lyse tumor cells in the absence of antibody. Pharmacokinetic studies showed that the half-life of anti-HER2/*neu* IgG3-(GM-CSF) depended on the injected dose with longer *in vivo* persistence observed at higher doses. Biodistribution studies showed that anti-HER2/*neu* IgG3-(GM-CSF) is mainly localized in the spleen. In addition, anti-HER2/*neu* IgG3-(GM-CSF) was able to target the HER2/*neu* expressing murine tumor CT26-HER2/*neu* and enhance the immune response against the targeted antigen HER2/*neu*. Anti-HER2/*neu* IgG3-(GM-CSF) is able to enhance both Th1 and Th2 mediated immune responses and treatment with this antibody fusion protein resulted in significant retardation in the growth of s.c. CT26-HER2/*neu* tumors. Our results indicate that anti-HER2/*neu* IgG3-(GM-CSF) fusion protein is useful in the treatment of HER2/*neu* expressing tumors. Our manuscript entitled "A recombinant anti-human HER2/*neu* IgG3-(GM-CSF) fusion protein retains antigen specificity, cytokine function and demonstrates anti-tumor activity" by Dela Cruz J.S., Trinh K.R., Morrison S.L., and Penichet M.L. was published by Journal of Immunology (3). It was the first report describing the *in vitro* and *in vivo* properties anti-HER2/*neu* IgG3-(GM-CSF) fusion protein (see appendix).

During the time or this award we also completed the construction, expression and characterization (*in vitro* and *in vivo*) of an anti-HER2/*neu* IgG3-(IL-2) fusion protein (4). Like anti-HER2/*neu* IgG3-(GM-CSF), anti-HER2/*neu* IgG3-(IL-2) expressed in myeloma cells was correctly assembled and secreted and retained both antibody and cytokine activity. Treatment of immunocompentent mice with this antibody fusion protein resulted in significant retardation in the subcutaneous (s.c.) growth of CT26-HER2/*neu* tumors suggesting that anti-HER2/*neu* IgG3-(IL-2) fusion protein will be useful in the treatment of HER2/*neu* expressing tumors. Our manuscript entitled "A recombinant IgG3-(IL-2) fusion protein for the treatment of human HER2/*neu* expressing tumors" by Penichet M.L., Dela Cruz J.S., Shin S.U., and Morrison S.L was published by Human Antibodies (4). It was the first report describing the *in vitro* and *in vivo* properties anti-HER2/*neu* IgG3-(IL-2) fusion protein (see appendix).

During the time or this award we have also expanded our research to study the ability of our anti-HER2/*neu* antibody-cytokine fusion proteins to function as adjuvants in mice vaccinated with a soluble form of HER2/*neu* antigen (ECD^{HER2}). DNA and peptide based vaccines targeting the tumor antigen HER2/*neu* have elicited protection in animal models challenged with HER2/*neu* expressing cancers. However, protein vaccines consisting of the extracellular domain of HER2/*neu* (ECD^{HER2}) have not shown similar efficacy. To determine if anti-human HER2/*neu* antibody-cytokine fusion proteins containing IL-2, IL-12 or GM-CSF can act as adjuvants for ECD^{HER2} vaccination, mice were vaccinated with human ECD^{HER2}, ECD^{HER2} with anti-HER2/*neu* antibody IgG3, or ECD^{HER2} with each antibody-cytokine fusion proteins and challenged with TUBO, a syngeneic carcinoma expressing rat HER2/*neu*. Significant retardation of tumor growth and long-term survivors were observed in mice vaccinated with ECD^{HER2} plus all three antibody-cytokine fusion proteins. ECD^{HER2} plus IgG3-(GM-CSF) and ECD^{HER2} plus IgG3-(IL-2) vaccinated mice showed the highest anti-ECD^{HER2} humoral response with increased IgG1 and modest IgG2a levels. In contrast ECD^{HER2} plus IgG3-(IL-12) vaccinated mice showed the highest levels of IgG2a, with no increase in IgG1. Immune sera inhibited the *in vitro* growth of SK-BR-3 (a human breast cancer overexpressing HER2/*neu*), the transfer of immune sera into mice challenged with TUBO, lead to partial inhibition of tumor growth. Splenocytes from mice vaccinated with ECD^{HER2} plus IgG3-(GM-CSF) incubated with ECD^{HER2} demonstrated significant proliferation and IFN-γ secretion. Our results suggest that both humoral and cell-mediated responses may contribute to the elicited anti-tumor activity. Our studies suggest that anti-HER2/*neu* antibody-cytokine fusion proteins may be effective prophylactic and therapeutic agents against HER2/*neu* expressing tumors in patients. Importantly, patients with high levels of circulating shed ECD^{HER2} may benefit. Based on our results a manuscript entitled "Protein vaccination with the HER2/*neu* extracellular domain plus anti-HER2/*neu* antibody-cytokine fusion proteins induces a protective anti-HER2/*neu* immune response in mice" by Dela Cruz J.S., Lau S.Y., Ramirez E.M., De Giovanni C., Forni G., Morrison S.L., and Penichet M.L. (5) has been submitted. This manuscript is the first report describing an effective strategy to enhance the immune response against soluble HER2/*neu* using antibody-cytokine fusion proteins (see appendix).

To evaluate the immunological efficacy of the proposed antibody-cytokine fusion proteins, it is critical that tumors expressing the target antigen can grow in immunologically intact mice. To produce murine tumors expressing human HER2/*neu* on their surface, we transduced the murine cell lines CT26 (syngeneic to BALB/c), MC38 and EL4 (both syngeneic to C57BL/6) with a retroviral construct containing the cDNA encoding human HER2/*neu*. As explained in our original grant proposal, these three human-HER2/*neu* expressing cells were similar to their respective parental cell lines in histology and kinetics of tumor growth in subcutaneous space and more important, they grow *in vivo* while maintaining the expression of human HER2/*neu*. The availability of different murine cell lines expressing human HER2/*neu* makes it possible to

evaluate the effectiveness of HER2/*neu* targeted approaches in different cell lines and/or mouse strains. This is a very important issue because clear differences in the response to the same anti-cancer therapy are seen with different tumors and in different strains. For this reason, during the time of this award we expanded our repertoire of human HER2/*neu* expressing murine cell lines by transducing the murine B cell lymphoma 38C13 (syngenic to C3H/HeN) with the cDNA encoding human HER2/*neu*. We studied the *in vivo* properties of this new tumor model (38C13-HER2/*neu*) and found that this new model differs in its behavior from our previously developed human HER2/*neu* expressing murine tumors (CT26-HER2/*neu*, MC38-CT26-HER2/*neu* and EL4-CT26-HER2/*neu*). Our manuscript entitled "A murine B cell lymphoma expressing human HER2/*neu* undergoes spontaneous tumor regression and elicits anti-tumor immunity" by Penichet M.L., Dela Cruz J.S., Challita-Eid P.M., Rosenblatt J.D., and Morrison S.L. was published by Cancer Immunology and Immunotherapy (6) (see appendix).

LIST OF PERSONNEL

Sherie L. Morrison, PI
Manuel L. Penichet, co-PI
Kham (Ryan) Trinh, Research Specialist
Jay Dela Cruz, Graduate Student Researcher
Patrick Ng, Graduate Student Researcher

KEY RESEARCH ACCOMPLISHMENTS

1) The construction, expression and characterization of anti-human HER2/*neu* IgG3-(GM-CSF) fusion protein.

2) The development and characterization of a new human HER2/*neu* expressing murine tumor model, the 38C13-HER2/*neu*.

3) The definition of the mechanism of action of anti-HER2/*neu* IgG3-(IL-12) fusion proteins.

4) The construction, expression and initial *in vivo* characterization of anti-human HER2/*neu* IgG3-(IL-2).

5) The study and characterization of the three anti-HER2/*neu* fusion protein [IgG3-(GM-CSF), IgG3-(IL-12), and IgG3-(IL-2)] as enhancers of immune response against the extracellular domain of human HER2/*neu* antigen (ECD^{HER2}).

REPORTABLE OUTCOMES

Our research funded by the U.S. Army award has resulted in five original research manuscripts, two review manuscripts, one book chapter, three abstracts, and one patent as described below. In all our publications and presentations the Department of Defense Breast Cancer Research Program Grant has been duly acknowledged. Although the invention (patent application) was assigned to "The Regents of the University of California", the patent application clearly states "this invention was made with Government support under the grant DAMD17-99-1-9098 awarded by the Army. The Government has certain rights in this invention".

Original Articles:

1) Dela Cruz J.S., Trinh K.R., Morrison S.L., and Penichet M.L. Recombinant anti-human HER2/*neu* IgG3-(GM-CSF) fusion protein retains antigen specificity, cytokine function, and demonstrates anti-tumor activity. Journal of Immunology, 2000, 165 (9): 5112-5121.

2) Penichet M.L., Dela Cruz J.S., Challita-Eid P.M., Rosenblatt J.D., and Morrison S.L. A murine B cell lymphoma expressing human HER2/*neu* undergoes spontaneous tumor regression and elicits anti-tumor immunity. Cancer Immunology and Immunotherapy, 2001, 49 (12): 649-662.

3) Penichet M.L., Dela Cruz J.S., Shin S.U., and Morrison S.L. A recombinant IgG3-(IL-2) fusion protein for the treatment of human HER2/*neu* expressing tumors. Human Antibodies, 2001, 10 (1): 43-49.

4) Peng L, Penichet M.L., Dela Cruz J.S., Sampogna S.L., and Morrison S.L. Mechanism of antitumor activity of a single-chain IL-12 IgG3 antibody fusion protein (mscIL-12.her2.IgG3). Journal of Interferon and Cytokine Research. 2001, 21 (9): 709-720.

5) Dela Cruz J.S., Lau S.Y., Ramirez E.M., De Giovanni C., Forni G., Morrison S.L., and Penichet M.L. Protein vaccination with the HER2/*neu* extracellular domain plus anti-HER2/*neu* antibody-cytokine fusion proteins induces a protective anti-HER2/*neu* immune response in mice. Submitted.

Literature Reviews

1) Penichet M.L., and Morrison S.L. Antibody-cytokine fusion proteins for the therapy of cancer. Journal of Immunological Methods, 2001, 248 (1-2): 91-101.

2) Yoo E.M., Chintalacharuvu, K.R., Penichet M.L., and Morrison S.L. Myeloma expression system. Journal of Immunological Methods, 2002 261 (1-2): 1-20.

Book Chapter

1) Penichet M.L. and Morrison S.L. Antibody Engineering. In Encyclopedia of Molecular Medicine (EMM). Thomas E. Creighton, ed. John Wiley & Son, Inc., New York. 2002, Vol. 1, pp. 214-216.

Patent

1) Penichet M.L., Dela Cruz J.S., Peng L, and Morrison, S.L. Antibody fusion proteins: effective adjuvants of protein vaccination. U.S. application serial number: 10/118,473. Filed on April 5, 2002.

Abstracts:

1) Peng L.S., Penichet M.L., and Morrison S.L. *In vivo* anti-tumor activity of an anti-her-2/neu-IL12 antibody fusion protein. 91st Annual Meeting of the American Association for Cancer Research, San Francisco, California, USA, April 1-5, 2000. Proceedings of the American Association for Cancer Research 91th Annual Meeting, v.41, 2000, pg. 287.

2) Dela Cruz J.S., Trinh K.R., Shin S-U., Morrison S.L., and Penichet M.L. Recombinant antibody-(IL-2) and antibody-(GM-CSF) fusion proteins for the treatment of human HER2/*neu* expressing tumors. 92nd Annual Meeting of the American Association for Cancer Research, New Orleans, Louisiana, USA, March 24-28, 2001. Proceedings of the American Association for Cancer Research 92nd Annual Meeting, v.42, 2001, pg. 291.

3) Dela Cruz J.S., Ramirez E.M., Lau S.Y., De Giovanni C., Forni G., Morrison S.L., and Penichet M.L. Anti-HER2/*neu* antibody fusion proteins: effective enhancers of extracellular domain HER2/*neu* protein vaccination. 93rd Annual Meeting of the American Association for Cancer Research, San Francisco, California, USA, April 6-10, 2002. Proceedings of the American Association for Cancer Research 93rd Annual Meeting, v.43, pg. 1016.

Development of Cell Lines:

Three cell lines have been developed and characterized:

TEAV: it is the murine myeloma cell line P3X63Ag8.653 expressing and secreting anti-HER2/*neu* IgG3-(GM-CSF).

TAPW: it is the murine myeloma cell line P3X63Ag8.653 expressing and secreting anti-HER2/*neu* IgG3-(IL-2).

TAUT: it is the murine B cell lymphoma 38C13 expressing on its surface human HER2/*neu.*

TEAV, TAPW, and TAUT are names assigned in our laboratory following our nomenclature system. However, in publications we usually use a descriptive name for cell lines. We have found that by so doing the manuscript is much easier to read.

CONCLUSIONS

Our results suggest that the three antibody fusion proteins we have developed: anti-HER2/*neu* IgG3-(GM-CSF), anti-HER2/*neu* IgG3-(IL2), and anti-HER2/*neu* IgG3-(IL12) may be effective prophylactic and therapeutic agents against HER2/*neu* expressing tumors in human. The combination of an anti-HER2/*neu* antibody with GM-CSF, IL-2 or IL-12 yields a protein with the potential to eradicate tumor cells by a number of mechanisms including the down regulation of HER2/*neu* expression, ADCC and the stimulation of a strong anti-tumor immune response through the immunostimulatory activity of GM-CSF, IL-2 or IL-12. In the specific case of anti-HER2/*neu* IgG3-(IL12) the anti-angiogenic activity of IL-2 also contributes to the anti-tumor activity of this antibody fusion protein. In addition, the anti-HER2/*neu* antibody fusion protein may be effective against tumor cells which express a truncated form of ECD^{HER2} lacking the receptor function rendering them particularly resistant to anti-HER2/*neu* antibody therapy. Because of the ability of the cytokine's (GM-CSF, IL-2, and IL-12) to elicit an immune response to associated antigens, it is also possible that association of anti-HER2/*neu* IgG3-cytokine fusion proteins with soluble ECD^{HER2} shed by tumor cells will enhance the anti-tumor immune response.

More studies are required to define the optimal dose and injection schedule for our antibody fusion proteins, continue the study of mechanism of their anti-tumor activity, the potential side effects, and to explore it is effectiveness against other human HER2/*neu* expressing murine tumor models.

REFERENCES

1) Peng L, Penichet M.L., and Morrison S.L. A single chain IL-12 IgG3 antibody fusion protein retains antibody specificity and IL-12 bioactivity and demonstrates anti-tumor activity. Journal of Immunology, 1999, 163 (1): 250-258.

2) Peng L, Penichet M.L., Dela Cruz J.S., Sampogna S.L., and Morrison S.L. Mechanism of antitumor activity of a single-chain IL-12 IgG3 antibody fusion protein (mscIL-12.her2.IgG3). Journal of Interferon and Cytokine Research. 2001, 21 (9): 709-720.

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5) Dela Cruz J.S., Lau S.Y., Ramirez E.M., De Giovanni C., Forni G., Morrison S.L., and Penichet M.L. Protein vaccination with the HER2/*neu* extracellular domain plus anti-HER2/*neu* antibody-cytokine fusion proteins induces a protective anti-HER2/*neu* immune response in mice. Submitted.

6) Penichet M.L., Dela Cruz J.S., Challita-Eid P.M., Rosenblatt J.D., and Morrison S.L. A murine B cell lymphoma expressing human HER2/*neu* undergoes spontaneous tumor regression and elicits anti-tumor immunity. Cancer Immunology and Immunotherapy, 2001, 49 (12): 649-662.

APPENDICES

Our research funded by U.S. Army award has resulted in five original research manuscripts, two review manuscripts, one book chapter, three abstracts, and one patent. All are included in this section.

Original Articles:

1) Dela Cruz J.S., Trinh K.R., Morrison S.L., and Penichet M.L. Recombinant anti-human HER2/*neu* IgG3-(GM-CSF) fusion protein retains antigen specificity, cytokine function, and demonstrates anti-tumor activity. Journal of Immunology, 2000, 165 (9): 5112-5121.

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3) Penichet M.L., Dela Cruz J.S., Shin S.U., and Morrison S.L. A recombinant IgG3-(IL-2) fusion protein for the treatment of human HER2/*neu* expressing tumors. Human Antibodies, 2001, 10 (1): 43-49.

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6) Penichet M.L., and Morrison S.L. Antibody-cytokine fusion proteins for the therapy of cancer. Journal of Immunological Methods, 2001, 248 (1-2): 91-101.

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Book Chapter

8) Penichet M.L. and Morrison S.L. Antibody Engineering. In Encyclopedia of Molecular Medicine (EMM). Thomas E. Creighton, ed. John Wiley & Son, Inc., New York. 2002, Vol. 1, pp. 214-216.

Abstracts:

9) Peng L.S., Penichet M.L., and Morrison S.L. *In vivo* anti-tumor activity of an anti-her-2/neu-IL12 antibody fusion protein. 91st Annual Meeting of the American Association for Cancer Research, San Francisco, California, USA, April 1-5, 2000. Proceedings of the American Association for Cancer Research 91th Annual Meeting, v.41, 2000, pg. 287.

10) Dela Cruz J.S., Trinh K.R., Shin S-U., Morrison S.L., and Penichet M.L. Recombinant antibody-(IL-2) and antibody-(GM-CSF) fusion proteins for the treatment of human HER2/*neu* expressing tumors. 92nd Annual Meeting of the American Association for Cancer Research, New Orleans, Louisiana, USA, March 24-28, 2001. Proceedings of the American Association for Cancer Research 92nd Annual Meeting, v.42, 2001, pg. 291.

11) Dela Cruz J.S., Ramirez E.M., Lau S.Y., De Giovanni C., Forni G., Morrison S.L., and Penichet M.L. Anti-HER2/*neu* antibody fusion proteins: effective enhancers of extracellular domain HER2/*neu* protein vaccination. 93rd Annual Meeting of the American Association for Cancer Research, San Francisco, California, USA, April 6-10, 2002. Proceedings of the American Association for Cancer Research 93rd Annual Meeting, v.43, pg. 1016.

Patent

12) Penichet M.L., Dela Cruz J.S., Peng L, and Morrison, S.L. Antibody fusion proteins: effective adjuvants of protein vaccination. U.S. application serial number: 10/118,473. Filed on April 5, 2002.

Recombinant Anti-Human HER2/*neu* IgG3-(GM-CSF) Fusion Protein Retains Antigen Specificity and Cytokine Function and Demonstrates Antitumor Activity[1]

Jay S. Dela Cruz, K. Ryan Trinh, Sherie L. Morrison, and Manuel L. Penichet[2]

Anti-HER2/*neu* therapy of human HER2/*neu*-expressing malignancies such as breast cancer has shown only partial success in clinical trials. To expand the clinical potential of this approach, we have genetically engineered an anti-HER2/*neu* IgG3 fusion protein containing GM-CSF. Anti-HER2/*neu* IgG3-(GM-CSF) expressed in myeloma cells was correctly assembled and secreted. It was able to target HER2/*neu*-expressing cells and to support growth of a GM-CSF-dependent murine myeloid cell line, FDC-P1. The Ab fusion protein activated J774.2 macrophage cells so that they exhibit an enhanced cytotoxic activity and was comparable to the parental Ab in its ability to effect Ab-dependent cellular cytotoxicity-mediated tumor cell lysis. Pharmacokinetic studies showed that anti-HER2/*neu* IgG3-(GM-CSF) is stable in the blood. Interestingly, the half-life of anti-HER2/*neu* IgG3-(GM-CSF) depended on the injected dose with longer in vivo persistence observed at higher doses. Biodistribution studies showed that anti-HER2/*neu* IgG3-(GM-CSF) is mainly localized in the spleen. In addition, anti-HER2/*neu* IgG3-(GM-CSF) was able to target the HER2/*neu*-expressing murine tumor CT26-HER2/*neu* and enhance the immune response against the targeted Ag HER2/*neu*. Anti-HER2/*neu* IgG3-(GM-CSF) is able to enhance both Th1- and Th2-mediated immune responses and treatment with this Ab fusion protein resulted in significant retardation in the growth of s.c. CT26-HER2/*neu* tumors. Our results suggest that anti-HER2/*neu* IgG3-(GM-CSF) fusion protein is useful in the treatment of HER2/*neu*-expressing tumors. *The Journal of Immunology,* **2000, 165: 5112–5121.**

The *HER2/neu* protooncogene (also known as *c-erbB-2*) encodes a 185-kDa transmembrane glycoprotein receptor known as HER2/*neu* or $p185^{HER2}$ that has partial homology with the epidermal growth factor receptor and shares with that receptor intrinsic tyrosine kinase activity (1–3). It consists of three domains: a cysteine-rich extracellular domain; a transmembrane domain; and a short cytoplasmic domain (1–3). Overexpression of HER2/*neu* is found in 25–30% of human breast cancer and this overexpression is an independent predictor of both relapse-free and overall survival in breast cancer patients (4–7). Overexpression of HER2/*neu* also has prognostic significance in patients with ovarian (5), gastric (8), endometrial (9), and salivary gland cancers (10). The increased occurrence of visceral metastasis and micrometastatic bone marrow disease in patients with HER2/*neu* overexpression has suggested a role for HER2/*neu* in metastasis (11, 12).

The elevated levels of the HER2/*neu* protein in malignancies and the extracellular accessibility of this molecule make it an excellent tumor-associated Ag (TAA)[3] for tumor-specific therapeutic agents. In fact, treatment of patients with advanced breast cancer using the anti-HER2/*neu* Ab, trastuzumab (Herceptin, Genentech, San Francisco, CA), previously known as rhumAb HER2, directed at the extracellular domain of HER2/*neu* (ECD^{HER2}) (13), can lead to an objective response in some patients with tumors overexpressing the HER2/*neu* oncoprotein (14, 15). However, only a subset of patients shows an objective response (5 of the 43 (11.6%)) (14, 15). Although combination of trastuzumab with chemotherapy enhances its antitumor activity (9 of 37 patients with no complete response (24.3%)) (16), improved therapies are still needed for the treatment of HER2/*neu*-expressing tumors.

GM-CSF is a cytokine associated with the growth and differentiation of hemopoietic cells. It is also a potent immunostimulator with pleiotropic effects, including the augmentation of Ag presentation in a variety of cells (17–22), increased expression of MHC class II on monocytes and adhesion molecules on granulocytes and monocytes (23–25), and amplification of T cell proliferation (26). In animals, the injection of GM-CSF potentiates the protective effects of an antitumor vaccine by enhancing T cell immunity (26), and vaccination with GM-CSF-transduced cells has been shown to be effective in the treatment of experimental tumors in murine models (27–30).

Studies suggest that for GM-CSF to be effective it must be concentrated in the vicinity of the tumor, where it acts in a paracrine manner. A completed phase I clinical trial showed that vaccination of patients with metastatic melanoma with irradiated autologous melanoma cells engineered to secrete human GM-CSF-stimulated potent antitumor immunity (31). Although the results suggest that

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[3] Abbreviations used in this paper: TAA, tumor-associated Ag; DNS, *N,N*-dimethyl-1-aminonaphthalene-5-sulfonyl chloride (dansyl); rmGM-CSF, recombinant murine GM-CSF; ECD^{HER2}, extracellular domain of HER2/*neu* Ag; AP, alkaline phosphatase; ADCC, Ab-dependent cellular cytotoxicity; %ID/g tissue, percent of injected dose per gram of tissue.

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this immunization strategy has potential application in the treatment of minimal residual disease, the ex vivo genetic modification and reintroduction of cells into patients is limited by its patient-specific nature. Additionally, it is technically difficult, time consuming, and expensive to expand primary autologous human tumor cells to the numbers required for vaccination (31–34). Although in vivo gene delivery using viral vectors has been considered, the low transfer efficiency of retroviral vectors and the immunogenicity of adenoviral vectors have limited efficacy (34). Although systemic administration of GM-CSF is an alternative approach, patients in clinical trials receiving high doses of GM-CSF have experienced severe toxic side effects (35) including a reported fatality (36), and no significant antitumor activity has been achieved. Thus, the challenge of developing an effective approach for achieving high local concentrations of GM-CSF remains.

Ab-(GM-CSF) fusion proteins that recognize TAAs provide one approach for achieving effective GM-CSF-mediated immune stimulation at the site of the tumor. In the present report, we characterize a novel Ab fusion protein, anti-HER2/*neu* IgG3-(GM-CSF) containing the variable region of the humanized anti-HER2/*neu* Ab, trastuzumab (Herceptin, Genentech, San Francisco, CA), and the murine GM-CSF. The properties of anti-HER2/*neu* IgG3-(GM-CSF) suggest that it may provide an effective alternative for the therapy of HER2/*neu*-expressing tumors.

Materials and Methods

Cell lines

CT26 is a murine colon adenocarcinoma that was induced in BALB/c mice by intrarectal injection of *N*-nitroso-*N*-methylurethane (37, 38). It was provided by Dr. Young Chul Sung (Pohang University of Science and Technology, Pohang, Korea). CT26-HER2/*neu* was developed in our laboratory by transduction of CT26 cells with the cDNA-encoding human HER2/*neu* (39). We previously showed that this cell line is able to grow in immunocompetent mice while maintaining the expression of human HER2/*neu* on its surface (39).

J774.2, a murine macrophage cell line was obtained from Dr. Mathew Scharff (Albert Einstein College of Medicine, Bronx, NY). The P3X63Ag8.653 mouse nonproducing myeloma was purchased from the American Type Culture Collection (ATCC, Manassas, VA). These four cell lines (CT26, CT26-HER2/*neu*, J774.2, and P3X63Ag8.653) were cultured in IMDM supplemented with 5% bovine calf serum, L-glutamine, penicillin, and streptomycin. The GM-CSF-dependent murine myeloid cell line, FDC-P1, purchased from the ATCC, was cultured in IMDM supplemented with 10% FBS containing 25% WEHI-3-conditioned medium, L-glutamine, penicillin, and streptomycin. All cells were incubated at 37°C in the presence of 5% CO_2.

Mice

Female BALB/c mice 6–8 wk of age obtained from Taconic Farms (Germantown, NY) were used. All experiments were performed according to published procedures (40). Animals were housed in a facility using autoclaved polycarbonate cages containing wood shaving bedding. The animals received food and water ad libitum. Artificial light was provided under a 12/12-h light/dark cycle. The temperature of the facility was 20°C with 10–15 air exchanges per hour.

*Vector construction, transfection, and initial characterization of anti-human HER2/*neu *IgG3-C_H3-(GM-CSF)*

The DNA encoding the variable light (V_L) and heavy (V_H) chain domains of the humanized Ab hum4D5-8 (13) (15) or rhuMAb HER2 (14, 16) (generously provided by Paul Carter, Genentech) had previously been cloned into mammalian expression vectors for human κ light chain and IgG3 heavy chain, respectively (41). The mature form of murine GM-CSF was amplified from the plasmid pCEP4/GM-CSF generously provided by Dr. Mi-Hua Tao (Institute of Biomedical Sciences, Academia Sinica, Taipei, Taiwan) by PCR using the sense primer 5′-CCCCTCGCGAGCGCACCCAC CCGCTCACCC-3′ and the antisense primer 5′-CCGAATTCGTTAAC CTTTTTGGACTGGTTTTTTGCATTC-3′.

The PCR product was digested with *NruI*/*Eco*RI and cloned in the vector pAT3462 (previously developed in our laboratory) digested with *SspI*/*Eco*RI, yielding the vector pAT1791 (Fig. 1). The plasmids pAT6611, pAH4874 (both previously developed in our laboratory), and pAT1791 were digested with *Eco*RV/NsiI, *Eco*RV/*Bam*HI, and *NsiI*/*Bam*HI, respectively. The fragments containing the DNA encoding for anti-HER2/*neu* V_H and γ3 constant regions (from pAT6611), the expression vector backbone (from pAH4874), and GM-CSF (from pAT1791) were purified using a Qiagen (Chatsworth, CA) Gel Extraction Kit after electrophoresis in an 0.8% agarose gel. The three fragments were ligated, yielding the anti-human HER2/*neu* IgG3-C_H3-(GM-CSF) heavy chain expression vector pAH1792. A cell line that produces high levels of anti-human HER2/*neu* κ light chain, TAOL 5.2.3, was first obtained by transfecting P3X63Ag8.653 by electroporation with the mammalian expression vector for human anti-human HER2/*neu* κ (Fig. 1) and selecting resistant mycophenolic acid-stable transfectants. These were screened for L-chain secretion by ELISA (42). The heavy chain expression vector pAH1792 was used to electroporate the light chain producer TAOL 5.2.3 (Fig. 1). Stable transfectants were selected with 5 mM histidinol (Sigma, St. Louis, MO) and screened by ELISA for the secretion of heavy chain (42). Transfectants were biosynthetically labeled with [^{35}S]methionine (ICN, Irvine, CA), and the fusion protein was immunoprecipitated using rabbit anti-human IgG and a 10% suspension of staphylococcal protein A (IgGSorb, The Enzyme Center, Malden, MA) and analyzed by SDS-PAGE with or without reduction by β-ME. The fusion protein was purified from culture supernatants using protein A immobilized on Sepharose 4B fast flow (Sigma). Protein concentrations were determined by bicinchoninic acid-based protein assay (BCA Protein Assay; Pierce, Rockford, IL) and ELISA. Purity and integrity were assessed by Coomassie blue staining of proteins separated by SDS-PAGE. The potential presence of aggregates in the purified protein was studied by fast protein liquid chromatography (Superose 6, Amersham Pharmacia Biotech, Piscataway, NJ) in filtered and degassed PBS + 0.02% sodium azide.

Ag binding

CT26 or CT26-HER2/*neu* (10^6) cells were incubated with 1 μg anti-HER2/*neu* IgG3-(GM-CSF) in 0.1 ml PBS plus 2% of bovine calf serum for 2 h at 4°C. Recombinant anti-HER2/*neu* IgG3 (41) and recombinant anti-DNS IgG3 Abs were used as positive and negative isotype-matched controls, respectively. Cells were washed and incubated for 2 h at 4°C with 0.5 μg biotinylated goat anti-human IgG (PharMingen, San Diego, CA) in a volume of 0.1 ml of PBS plus 2% bovine calf serum. Cells were washed and incubated for 30 min with 0.03 μg PE-labeled streptavidin (PharMingen) in a volume of 0.1 ml PBS plus 2% of bovine calf serum. Analysis was performed by flow cytometry with a FACScan (Becton Dickinson, Mountain View, CA) equipped with a blue laser excitation of 15 mW at 488 nm.

Proliferation assay

The GM-CSF-dependent murine myeloid cell line FDC-P1 was used to study the bioactivity of anti-HER2/*neu* IgG3-(GM-CSF). rmGM-CSF from *Escherichia coli* with $ED_{50} \leq 0.2$ ng/ml (Chemicon, Temecula, CA) reconstituted using deionized water following the manufacturer's recommendations and stored at −20°C was used as reference standard. Serial 1:2 dilutions of equivalent molar concentrations of rmGM-CSF and anti-HER2/*neu* IgG3-(GM-CSF) were made in RPMI 1640 + 10% FBS, over a range of 2 ng/ml to 16 pg/ml. Similarly, serial 1:2 dilutions of control anti-HER2/*neu* IgG3 were also included with a concentration equivalent to the Ab portion of anti-HER2/*neu* IgG3-(GM-CSF). 50 μl (5000 cells/well) FDC-P1 myeloid cells in RPMI 1640 + 10% FBS were mixed with 50 μl serial dilutions of rmGM-CSF, anti-HER2/*neu* IgG3-(GM-CSF), anti-HER2/*neu* IgG3, or medium in quadruplicate in a flat-bottom 96-well tissue culture plate (Costar, Corning, NY). After 48 h of culture at 37°C, 5% CO_2, proliferation was measured using the Cell Titer 96 aqueous nonradioactive colorimetric assay (Promega, Madison, WI), and plates were read at 490 nm.

Macrophage-mediated cytotoxicity

Macrophage-mediated cytotoxicity was performed according to the methods of Duerst and Werberig (43) using the DNA fragmentation assay of Matzinger (44) with modifications. Briefly, the target cells CT26-HER2/*neu* were labeled with [^{3}H]thymidine (ICN) at 5 μCi/ml (sp act 6.7 Ci/mmol) in IMDM supplemented with 5% bovine calf serum for 24 h at 37°C. Labeled target cells were washed with medium and incubated with J774.2 macrophage effector cell in the presence of 5 μg/ml anti-HER2/*neu* IgG3, the molar equivalent amount of anti-HER2/*neu* IgG3-(GM-CSF) or no Ab for 24 h at 37°C. Alternatively, J774.2 cells were incubated with 6.72×10^{-2} μg/ml anti-HER2/*neu* IgG3-(GM-CSF) (equivalent to 50

U/ml GM-CSF portion of anti-HER2/*neu* IgG3-(GM-CSF)), with anti-HER2/*neu* IgG3 at a concentration equivalent to the Ab portion of anti-HER2/*neu* IgG3-(GM-CSF) (5.68×10^{-2} μg/ml), or with no additions in IMDM supplemented with 5% bovine calf serum for 24 h at 37°C. After incubation, the J774.2 cells were washed with medium and then transferred into a 96-well round-bottom tissue culture plate (Costar) containing 1×10^4 [^{3}H]thymidine-labeled CT26-HER2/*neu* per well (E:T 10). All incubations were conducted for 24 h in a final volume of 200 μl/well using IMDM supplemented with 5% bovine calf serum and 50 μM cold thymidine. The presence of 50 μM cold thymidine blocks the incorporation of released [^{3}H]thymidine by the J774.2 effector cells (43). The cells were harvested and passed through a glass-fiber filter (Wallac Oy, Turku, Finland) using a Micro Cell Harvester (Skatron, Lier, Norway). Labeled DNA from intact target cells was captured by the filters. The radioactivity was measured with a 1205 Betaplate Liquid Scintillation Counter (Wallac Oy, Turku, Finland). The percent cytotoxicity mediated by J774.2 macrophage cells was calculated by the formula: [(cpm control − cpm test)/cpm control)] × 100; where cpm control represents ^{3}H measured in the wells containing target cells and anti-HER2/*neu* IgG3, anti-HER2/*neu* IgG3-(GM-CSF), or medium but lacking J774.2 macrophage cells. cpm test represents wells containing target cells in the presence of either effector cells preincubated with anti-HER2/*neu* IgG3 or anti-HER2/*neu* IgG3-(GM-CSF) or neither and Abs (anti-HER2/*neu* IgG3 or anti-HER2/*neu* IgG3-(GM-CSF)). All assays were done in quadruplicate.

Half-life

Anti-HER2/*neu* IgG3-(GM-CSF) was iodinated to ~2 μCi/μg with ^{125}I using Iodo-Beads (Pierce) according to manufacturer's protocol. Mice were injected i.v. via the lateral tail vein with 1 μCi ^{125}I-labeled proteins alone or mixed with 20 μg cold anti-HER2/*neu* IgG3-(GM-CSF). At various intervals after injection of ^{125}I-labeled anti-HER2/*neu* IgG3-(GM-CSF), residual radioactivity was measured using a mouse whole body counter (Wm. B. Johnson, Montville, NJ). Blood samples were obtained from the tail vein of mice 2, 4, and 12 h after injection. Serum was separated from clotted blood and stored at −20°C until assayed by SDS-PAGE to confirm the integrity of the protein.

Biodistribution

Groups of 4 mice were sacrificed 4 or 16 h after the i.v. injection of 1 μCi (0.5 μg) ^{125}I-labeled anti-HER2/*neu* IgG3-(GM-CSF). Various organs and blood were collected and weighed, and radioactivity was measured using a gamma counter (Gamma 5500, Beckman Coulter, Fullerton, CA). Data are presented as percent of injected dose per gram of tissue (%ID/g tissue). Values were corrected for the radioactivity in blood in each tissue using the values of blood volume corresponding to each organ (45).

Tumor targeting

Anti-HER2/*neu* IgG3-(GM-CSF) was iodinated as described above. CT26 and CT26-HER2/*neu* cells (10^6 in 0.15 ml HBSS (Life Technologies, Grand Island, NY)) were injected separately into the left and right flanks of three mice. Seven days after tumor injection when tumors were ~1.0 cm in diameter, the three mice were injected i.v. via the lateral tail vein with 6 μCi ^{125}I-labeled anti-HER2/*neu* IgG3-(GM-CSF). Mice were euthanized 12 h after injection of anti-HER2/*neu* IgG3-(GM-CSF). Tumors and blood were removed and weighed, and radioactivity was measured with a gamma counter. Data are presented as %ID/g tumor.

Immunotherapy

CT26-HER2/*neu* cells (1×10^6 in 0.15 ml HBSS) were injected s.c. into the right flank of syngeneic BALB/c mice. Beginning the next day, mice randomized into groups of eight received five daily i.v. injections of 0.25 ml PBS containing 20 μg anti-HER2/*neu* IgG3-(GM-CSF), the equivalent molar amount of anti-HER2/*neu* IgG3, or nothing. Tumor growth was monitored and measured with a caliper every 3 days until day 15 at which time mice were euthanized. Blood samples were collected, and serum was separated from clotted blood and stored at −20°C until assayed by ELISA.

*Determination of murine anti-human HER2/*neu *and anti-human IgG3 Abs*

Sera from each treatment group were analyzed by ELISA for the presence of Abs to human IgG3 and human HER2/*neu* using 96-well microtiter plates coated with 50 μl anti-human HER2/*neu* IgG3 or human ECD^{HER2} (at a concentration of 1 μg/ml), respectively. The plates were blocked with 3% BSA in PBS, and dilutions of serum in PBS containing 1% BSA were added to the wells and incubated overnight at 4°C. After a washing with PBS, alkaline phosphatase (AP)-labeled goat anti-mouse IgG (Sigma) was added, and the plates were incubated for 1 h at 37°C. After a washing, *p*-nitrophenyl phosphate disodium dissolved in diethanolamine buffer (Sigma) was added to the wells for 1 h, and plates were read at 410 nm. Sera from mice of the same age bearing tumors of the parental cell line CT26 was used as a negative control for determining anti-HER2/*neu* titers. Sera from naive mice of the same age were used as a negative control for determining anti-human IgG3 titers. All ELISAs for comparison of titers between the experimental groups were made simultaneously in duplicate using an internal positive control curve for each plate.

*Determination of isotype profile of murine anti-human HER2/*neu *and anti-human IgG3 Abs*

The isotype of the murine anti-human IgG3 and anti-human HER2/*neu* was determined by ELISA using 96-well microtiter plates prepared as described above. Pooled sera from each treatment group diluted 1:50 in 1% BSA in PBS was added at 50 μl/well in duplicate into the 96-well plates and allowed to stand overnight at 4°C. After the plates were washed with PBS, rat Abs specific for murine IgG2a, IgG2b, IgG3, IgG1, or κ (PharMingen) diluted in 1% BSA in PBS were added to each well and incubated 2 h at room temperature. After washing with PBS, alkaline phosphatase (AP)-labeled goat anti-rat IgG (PharMingen) was added, and the plates were processed as described above.

Statistical analysis

Statistical analysis of the titration ELISA was conducted using the Mann-Whitney rank test, and the statistical analysis of the DNA fragmentation assay and the antitumor experiments was done using a two-tailed Student *t* test. For all cases, results were regarded significant if p values were ≤ 0.05.

Results

*Construction, expression, and initial in vitro characterization of anti-HER2/*neu *IgG3-C_H3-(GM-CSF)*

The strategy for the construction and expression of anti-HER2/*neu* IgG3-C_H3-(GM-CSF) is illustrated in Fig. 1. Clones expressing anti-HER2/*neu* IgG3-C_H3-(GM-CSF) were identified by ELISA and biosynthetically labeled by growth in the presence of [^{35}S]methionine. Labeled secreted protein was immunoprecipitated using rabbit anti-human IgG and analyzed by SDS-PAGE under reducing and nonreducing conditions. The anti-HER2/*neu* IgG3-C_H3-(GM-CSF) was correctly assembled and secreted and exhibits the expected m.w. (data not shown). These results were confirmed by SDS-PAGE of purified proteins. In the absence of reducing agents anti-HER2/*neu* IgG3 migrates with an apparent molecular mass of 170 kDa whereas anti-HER2/*neu* IgG3-(GM-CSF) is ~200 kDa, the size expected for a complete IgG3 with 2 molecules of GM-CSF attached (Fig. 2*A*). After treatment with the reducing agent, light chains migrating with an apparent molecular mass of ~25 kDa are seen for both proteins. However, the anti-HER2/*neu* IgG3 has a heavy chain with an apparent molecular mass of ~60 kDa, whereas anti-HER2/*neu* IgG3-(GM-CSF) has a heavy chain with an apparent molecular mass of ~75 kDa (Fig. 2*B*) as expected. Thus, proteins of the expected molecular mass are produced and fusion of murine GM-CSF to the carboxyl terminus of the heavy chain of anti-HER2/*neu* IgG3 does not appear to alter the assembly and secretion of the H_2L_2 form of the Ab fusion protein. Analysis of anti-HER2/*neu* IgG3 and anti-HER2/*neu* IgG3-(GM-CSF) by fast protein liquid chromatography under nondenaturing conditions showed that both proteins eluted as a single peak of the expected m.w. with no evidence of aggregation (data not shown).

Ag binding at the cell surface

The ability of anti-HER2/*neu* IgG3-(GM-CSF) to bind to the HER2/*neu* target Ag was examined using flow cytometry. Both anti-HER2/*neu* IgG3-(GM-CSF) and anti-HER2/*neu* IgG3 specifically bound to the human HER2/*neu* expressed on the surface of the murine cell line CT26-HER2/*neu* (Fig. 3, *B* and *C*). The same

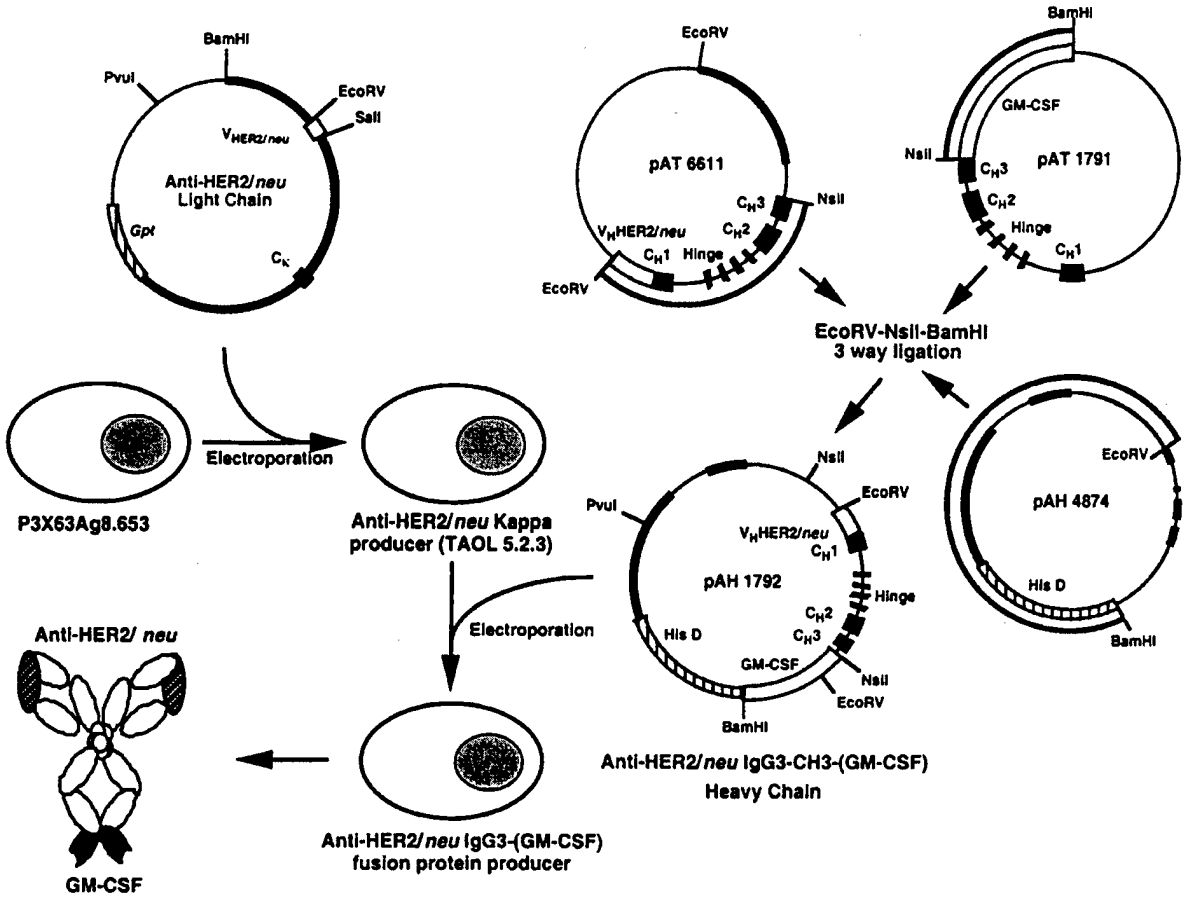

FIGURE 1. Construction and expression of anti-HER2/*neu* IgG3-(GM-CSF). The expression vector for anti-HER2/*neu* IgG3-(GM-CSF), pAH1792, was constructed by three-way ligation of the fragments containing the V_H anti-HER2/*neu* and constant IgG3 regions from pAT6611, the expression vector backbone from pAH4874, and GM-CSF from pAT1791. A solid line outside the plasmid indicates the fragment used in the three-way ligation. TAOL 5.2.3, a transfectant of P3X63Ag8.653 expressing a light chain with the anti-HER2/*neu* variable region, was used as a recipient for transfection of the anti-HER2/*neu* IgG3-(GM-CSF) heavy chain expression vector pAH1792.

fluorescence intensity was seen, which suggests that they have the same affinity for HER2/*neu*. No nonspecific binding to CT26 that does not express HER2/*neu* was observed (Fig. 3, *E* and *F*).

Proliferation assay

Anti-HER2/*neu* IgG3-(GM-CSF) was able to specifically stimulate the proliferation of the GM-CSF-dependent cell line FDC-P1. The proliferative response to equimolar GM-CSF concentrations of either rmGM-CSF or the anti-HER2/*neu* IgG3-(GM-CSF) fusion protein was similar (Fig. 4). No proliferation was detected when cells were incubated with the same amount of anti-HER2/*neu* IgG3 (data not shown). The GM-CSF activity of anti-HER2/*neu* IgG3-(GM-CSF) present in culture supernatants was similar to that of purified protein, indicating that the low pH used for elution from protein A does not reduce GM-CSF activity (data not shown).

Macrophage-mediated cytotoxicity

Two assays were used to examine the ability of anti-HER2/*neu* IgG3-(GM-CSF) to augment macrophage-mediated killing of tumor cells. Tumor cells and the macrophage cell line J774.2 were incubated for 24 h in the presence of 5 μg/ml anti-HER2/*neu* IgG3 or the molar equivalent of anti-HER2/*neu* IgG3-(GM-CSF). Equivalent tumor cell lysis was seen with both proteins, indicating that the Fc region of the fusion protein can be bound by the macrophage receptors to elicit ADCC (Fig. 5*A*). The tumor cell lysis observed with the incubation of anti-HER2/*neu* IgG3 or anti-HER2/*neu* IgG3-(GM-CSF) was statistically significant when compared with the results obtained with the incubation of the effector and target cells in absence of the Abs ($p < 0.05$). In the second assay, effector cells were incubated with 6.72×10^{-2} μg/ml anti-HER2/*neu* IgG3-(GM-CSF) or anti-HER2/*neu* IgG3, washed to remove unbound Ab or fusion protein, and then incubated with labeled target cells for 24 h. Anti-HER2/*neu* IgG3-(GM-CSF)-treated J774.2 cells were significantly ($p < 0.0002$) more effective in lysing tumor cells than the effector cells activated in presence of anti-HER2/*neu* IgG3 (Fig. 5*B*) which were similar to nonactivated effector cells added to labeled cells in the absence of Abs. Therefore, the GM-CSF in the fusion protein retains the ability to mediate macrophage activation.

Half-life

The half-life of ^{125}I-labeled anti-HER2/*neu* IgG3 and anti-HER2/*neu* IgG3-(GM-CSF) was examined in BALB/c mice. Mice were injected i.v. via the lateral tail vein with 1 μCi (0.5 μg) ^{125}I-labeled protein, and the residual radioactivity measured using a

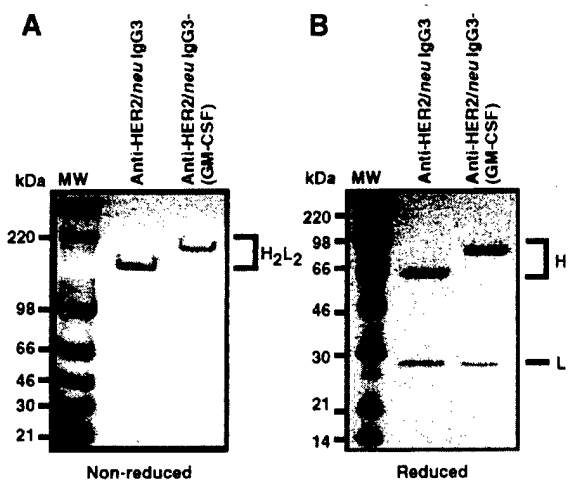

FIGURE 2. SDS-PAGE analysis of anti-HER2/*neu* IgG3-(GM-CSF). Secreted anti-HER2/*neu* IgG3-(GM-CSF) was purified from culture supernatants using protein A immobilized on Sepharose 4B fast flow and analyzed by SDS-PAGE under nonreducing (*A*) and reducing (*B*) conditions. Included for comparison is anti-HER2/*neu* IgG3 without attached GM-CSF. The positions of the m.w. standards are indicated at the left sides.

mouse whole body counter. Anti-HER2/*neu* IgG3 exhibited a half-life of 110 h, similar to what had previously been observed with chimeric IgG3 (46) (Fig. 6). Anti-HER2/*neu* IgG3-(GM-CSF) cleared more rapidly with a half-life of ~2 h, indicating that fusion of the murine GM-CSF to the human anti-HER2/*neu* IgG3 significantly decreases the half-life. However, because we plan to treat the mice with a much higher dose (20 μg) of anti-HER2/*neu* IgG3-(GM-CSF), we also studied the half-life when this amount of protein was injected by mixing 20 μg cold anti-HER2/*neu* IgG3-(GM-CSF) with 1 μCi (0.5 μg) ^{125}I-labeled anti-HER2/*neu* IgG3-(GM-CSF) before injection. Increasing the quantity of injected anti-HER2/*neu* IgG3-(GM-CSF) injected increased the half-life 5- to 6-fold (10–12 h) (Fig. 6). Although results shown in Fig. 6 represent the mean of only two mice per group, similar results were obtained when this experiment was repeated (data not shown).

Sera obtained from each mouse 2, 4, and 12 h after injection were fractionated without reduction on SDS-PAGE and examined by autoradiography. The radioactivity was present at the position expected for intact protein, with the intensity of the band correlating with the residual radioactivity determined by whole body counting.

Biodistribution

Groups of four mice injected i.v. via the lateral tail vein with 1 μCi ^{125}I-labeled anti-HER2/*neu* IgG3-(GM-CSF) were euthanized 4 h (time equivalent to two half-lives of the injected protein) or 16 h

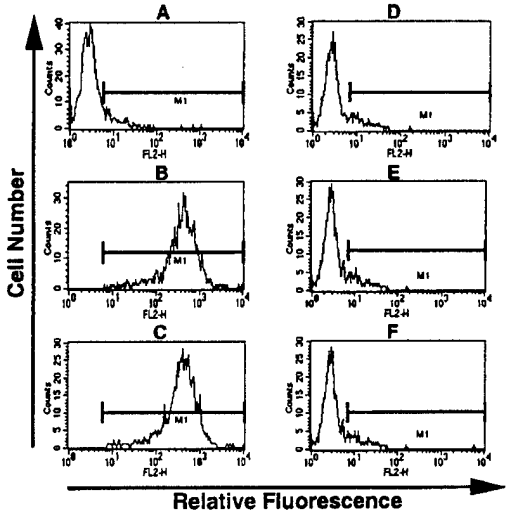

FIGURE 3. Flow cytometry demonstrating the specificity of anti-HER2/*neu* IgG3-(GM-CSF) for the HER2/*neu* expressed on the surface of CT26-HER2/*neu*. CT26-HER2/*neu* (*A–C*) or the non-HER2/*neu*-expressing parental cell line CT26 (*D–F*) were stained with anti-DNS human IgG3 (*A* and *D*), anti-HER2/*neu* human IgG3 (*B* and *E*) or anti-HER2/*neu* IgG3-(GM-CSF) (*C* and *F*), followed by biotinylated goat anti-human IgG and PE-labeled streptavidin. FL2-H, Fluorescence.

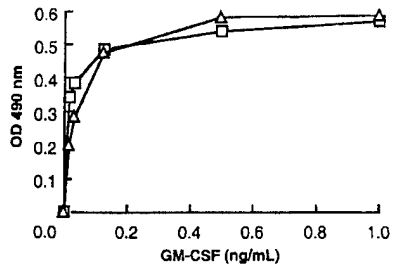

FIGURE 4. Bioactivity assay. FDC-P1 cells were incubated with various concentrations of rmGM-CSF (□) or anti-HER2/*neu* IgG3-(GM-CSF) (△). The concentration of anti-HER2/*neu* IgG3-(GM-CSF) was adjusted to the GM-CSF portion of the fusion protein obtaining equivalent molar concentrations of rmGM-CSF and anti-HER2/*neu* IgG3-(GM-CSF). Proliferation was measured by a colorimetric assay and read at 490 nm. All results are expressed as mean OD_{490} of quadruplicate wells with a SD of <20% for each concentration.

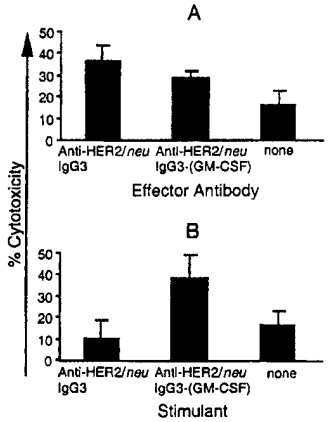

FIGURE 5. Macrophage-mediated cytotoxicity. *A*, A total of 1×10^4 ^{3}H-labeled CT26-HER2/*neu* target cells were cultured for 24 h with anti-HER2/*neu* IgG3 (5 μg/ml), the equivalent molar concentration of anti-HER2/*neu* IgG3-(GM-CSF), or nothing in the presence of J774.2 macrophage effector cells at an E:T ratio of 10. *B*, Effector cells were preincubated for 24 h with anti-HER2/*neu* IgG3-(GM-CSF) (6.72×10^{-2} μg/ml), the equivalent molar concentration of anti-HER2/*neu* IgG3, or nothing; washed; and then incubated with 1×10^4 ^{3}H-labeled CT26-HER2/*neu* target cells for 24 h. For both assays, intact DNA from live target cells was collected by a cell harvester, and radioactivity was measured using a scintillation counter. Bars represent the SD of quadruplicate samples.

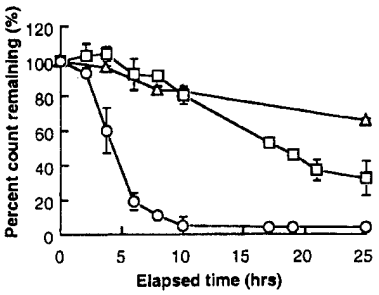

FIGURE 6. Half-life of anti-HER2/*neu* IgG3-(GM-CSF) and anti-HER2/*neu* IgG3. Groups of two mice were injected i.v. via the lateral tail vein with 1 μCi (0.5 μg) ^{125}I-labeled anti-HER2/*neu* IgG3 (△), anti-HER2/*neu* IgG3-(GM-CSF) (○), or 1 μCi (0.5 μg) ^{125}I-labeled anti-HER2/*neu* IgG3-(GM-CSF) mixed with 20 μg cold anti-HER2/*neu* IgG3-(GM-CSF) (□). At various intervals after injection of the ^{125}I-labeled protein, residual radioactivity was measured using a mouse whole body counter. The results represent the mean of two mice. Bars represent the range of values obtained.

after injection. Various organs and blood were collected and weighed, and radioactivity was measured using a gamma counter. Four hours after its injection anti-HER2/*neu* IgG3-(GM-CSF) shows targeting to the spleen, followed by the kidneys, liver, and lungs (Fig. 7*A*). By 16 h after the injection, most of anti-HER2/*neu* IgG3-(GM-CSF) had cleared with some radioactivity remaining in the spleen, kidneys, and blood. Splenic uptake may reflect the large number of GM-CSF receptor-bearing cells in this organ. The presence of radioactivity in the kidneys and liver, sites of degradation and elimination, is consistent with the rapid elimination of anti-HER2/*neu* IgG3-(GM-CSF).

Tumor targeting

To examine the tumor targeting capability of anti-HER2/*neu* IgG3-(GM-CSF), BALB/c mice were injected with 10^6 CT26 and CT26-HER2/*neu* tumor cells in the left and right flanks, respectively. Seven days after tumor injection when tumors were ~1.0 cm in diameter, groups of three mice were injected i.v. via the lateral tail vein with 6 μCi ^{125}I-labeled anti-HER2/*neu* IgG3-(GM-CSF). The mice were euthanized 12 h later, the tumors and blood were removed and weighed, and the ^{125}I-labeled protein present was measured by a gamma counter. In all mice, enhanced localization of ^{125}I-labeled anti-HER2/*neu* IgG3-(GM-CSF) was seen in the CT26-HER2/*neu* tumor compared with CT26 that did not express HER2/*neu* (Fig. 8). These data indicate that anti-HER2/*neu* IgG3-(GM-CSF) is able to specifically target HER2/*neu*-expressing cells.

Antitumor activity

To investigate in vivo antitumor activity, 10^6 CT26-HER2/*neu* cells were injected s.c. into the right flank of BALB/c mice. Beginning the next day, mice were randomized, and groups of eight received five daily i.v. injections of 0.25 ml PBS containing 20 μg anti-HER2/*neu* IgG3-(GM-CSF), the equivalent molar amount of anti-HER2/*neu* IgG3, or nothing. Injection of anti-HER2/*neu* IgG3-(GM-CSF) results in a significant retardation in the tumor growth in most of the mice as compared with the respective controls of PBS or anti-HER2/*neu* IgG3 (Fig. 9, Experiment 1). When the experiment was repeated similar results were obtained (Fig. 9, Experiment 2). When the data of Experiments 1 and 2 were pooled, treatment with anti-HER2/*neu* IgG3-(GM-CSF) was found to result in highly significant antitumor activity ($p \leq 0.02$) for all the observed points (Table I). There was no statistically significant difference in tumor volume between the groups injected with PBS and anti-HER2/*neu* IgG3.

*Murine Ab response to HER2/*neu *and human IgG3*

Sera from all mice in Experiment 2 were analyzed for the presence of Abs recognizing the TAA HER2/*neu* and the human IgG3 Ab used for treatment. Mice treated with anti-HER2/*neu* IgG3-(GM-CSF) exhibited a significantly increased Ab response to both HER2/*neu* ($p < 0.04$) and human IgG3 ($p < 0.001$) compared with mice treated with either PBS or anti-HER2/*neu* IgG3 (Table II).

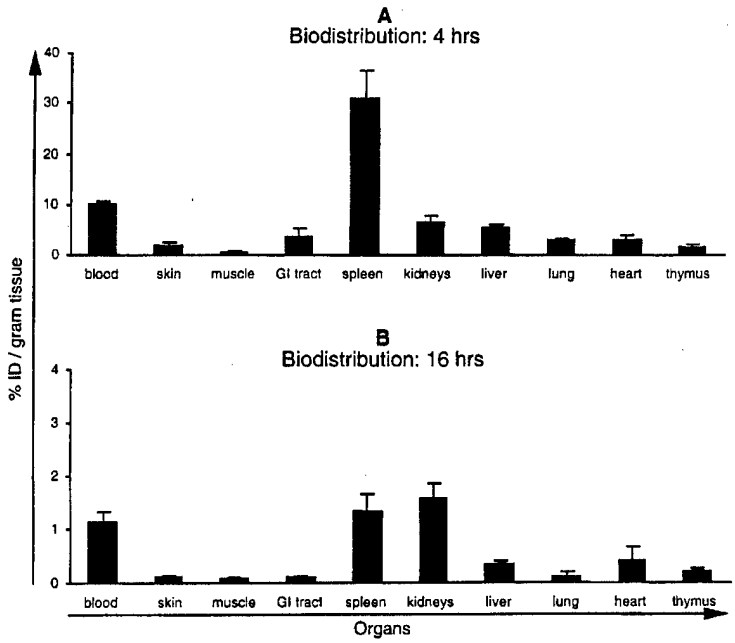

FIGURE 7. Biodistribution of anti-HER2/*neu* IgG3-(GM-CSF). Two groups of four mice were injected i.v. via the lateral tail vein with 1 μCi (0.5 μg) ^{125}I-labeled anti-HER2/*neu* IgG3-(GM-CSF), and mice were euthanized after 4 h, which is the equivalent of two half-lives for the injected dose or after 16 h. Various organs and blood were collected and weighed, and radioactivity was measured using a gamma counter. Data are presented as %ID/g tissue. GI, Gastrointestinal. Bars represent the SD of the data obtained.

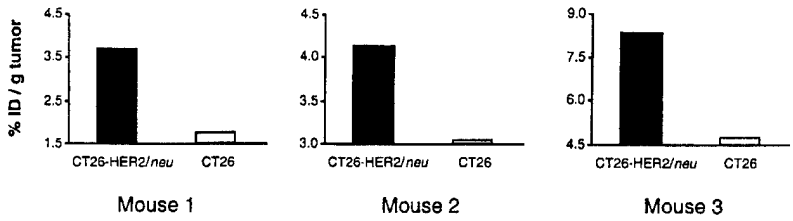

FIGURE 8. Tumor targeting of anti-HER2/*neu* IgG3-(GM-CSF). CT26-HER2/*neu* and CT26 cells (10^6) were injected separately into the right and left flanks of three BALB/c mice. After 1 wk, when the tumor diameter was ~1.0 cm, groups of three mice were injected i.v. via the lateral tail vein with ^{125}I-labeled anti-HER2/*neu* IgG3-(GM-CSF). Mice were euthanized 12 h after injection. Blood and tumors were collected and weighed, and radioactivity was measured by a gamma counter. Data are presented as %ID/g tumor.

Isotype of murine Ab response

To further characterize the Ab response, the relative levels of the different isotypes present in the serum of anti-HER2/*neu* IgG3-(GM-CSF) and anti-HER2/*neu* IgG3-treated mice were determined (Fig. 10). Mice treated with anti-HER2/*neu* IgG3-(GM-CSF) showed significantly higher levels of all isotypes (with the exception of IgG3) recognizing human IgG3 when compared with anti-HER2/*neu* IgG3 treated mice (Fig. 10*A*). The increase in Abs of the γ2a and γ1 isotypes suggests activation of both Th1- and Th2-mediated responses against this Ag, respectively. When Abs directed against HER2/*neu* were examined (Fig. 10*B*), animals treated with anti-HER2/*neu* IgG3-(GM-CSF) showed an increase in γ2b and γ1 but not γ3 and γ2a compared with animals treated with anti-HER2/*neu* IgG3. Thus, the increased Ab response to HER2/*neu* was predominantly of the isotypes characteristic of the Th2 response.

Discussion

In an attempt to improve the clinical efficacy of anti-HER2/*neu* based therapies, we have developed an alternative approach in which a human IgG3 containing the variable regions of trastuzumab (Herceptin, Genentech, San Francisco, CA) has been genetically fused to potent immunostimulatory molecules such as the cytokine IL-12 (47) and the costimulatory molecule B7.1 (41). In the present study, we expand this family of anti-HER2/*neu* Ab fusion proteins to include a fusion with the important cytokine GM-CSF.

A number of factors were considered in the design of our anti-HER2/*neu* IgG3-(GM-CSF) fusion protein. Human IgG3 was chosen because its extended hinge region should provide spacing and flexibility, thereby facilitating simultaneous Ag and receptor binding (48, 49). IgG3 is also effective in complement activation (50) and binds FcγRs (51). GM-CSF was used because of its potent immunostimulating properties and ability to serve as a strong potentiator of tumor vaccines (26–30). Although our long-term goal is the production of Ab fusion proteins for therapeutic use in humans, human GM-CSF is not active in mice (35). Therefore we used murine GM-CSF in our fusion protein so that we could perform in vivo studies using immune competent mice. We found that anti-HER2/*neu* IgG3-(GM-CSF) retains the ability to bind HER2/*neu* while the murine GM-CSF attached to the carboxyl terminus of each heavy chain remains active.

In addition to the Ab-induced down-regulation of HER2/*neu* expression ADCC has been proposed as a possible mechanism for the clinical response observed with trastuzumab (15). Indeed, recent studies have indicated that ADCC is an important effector mechanism for Ab-mediated tumor rejection (52). Fusion of GM-CSF to the carboxyl terminus of C_H3 did not interfere with the ability of Ab to mediate ADCC (Fig. 5*A*). In addition, preincubation of macrophages with a very low concentration of anti-HER2/*neu* IgG3-(GM-CSF) results in a significant activation of macrophage-mediated cytotoxicity as compared with anti-HER2/*neu* IgG3 (Fig. 5*B*). In this latter experiment Abs were not added to the E:T mixture, suggesting that preincubation of macrophage with anti-HER2/*neu* IgG3-(GM-CSF) results in the activation of ADCC. However, because the effector cells were preincubated with anti-HER2/*neu* IgG3-(GM-CSF), the possibility of ADCC mediated by Ab-coated effector cells cannot be excluded.

A recombinant fusion protein with a human-mouse chimeric IgG1 specific for B cell malignancies fused to human GM-CSF (chCLL-1/GM-CSF) showed enhanced ADCC activity using human mononuclear cells compared with Ab (chCLL-1) alone (53). It is therefore possible that an anti-HER2/*neu* IgG3-(GM-CSF) containing human GM-CSF will exhibit superior antitumor activity. In addition directing GM-CSF to the tumor microenvironment

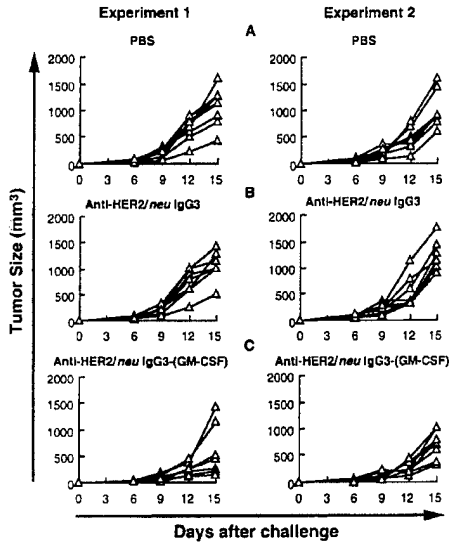

FIGURE 9. Antitumor activity of anti-HER2/*neu* IgG3-(GM-CSF) and anti-HER2/*neu* IgG3. 10^6 CT26-HER2/*neu* cells were injected s.c. into the right flank of BALB/c mice. Beginning the next day, groups of eight mice received five daily i.v. injections of 0.25 ml PBS containing 20 μg anti-HER2/*neu* IgG3-(GM-CSF), the equivalent molar amount of anti-HER2/*neu* IgG3, or nothing. Tumor growth was measured with a caliper every 3 days until day 15. The volume was calculated for each mouse of each treatment group, PBS (*A*), anti-HER2/*neu* IgG3 (*B*), and anti-HER2/*neu* IgG3-(GM-CSF) (*C*). Experiments 1 and 2 were conducted under identical conditions but at different time.

Table I. *Mean tumor volumes and statistical significance*

Days After Challenge	Mean Tumor Volumes[a]			Significance[b]	
	PBS	IgG3	IgG3-(GM-CSF)	(*p*) 1	(*p*) 2
6	60.8	71	37.6	0.02	0.0006
9	211	224.5	110.5	0.0008	0.0003
12	578.2	631.8	264.9	0.0001	0.0001
15	1041.8	1155.6	655.3	0.0053	0.0002

[a] CT26-HER2/*neu* cells (10^6) were injected s.c. into the right flank of BALB/c mice. Beginning the next day, groups of eight mice received five daily i.v. injections of 0.25 ml PBS containing 20 μg anti-HER2/*neu* IgG3-(GM-CSF), the equivalent molar amount of anti-HER2/*neu* IgG3, or nothing. Tumor growth was measured with a caliper every 3 days until day 15, and the volume was calculated for each mouse of each treatment group. The experiment was conducted twice under identical conditions. Mean tumor volumes represents the average tumor volume for each treatment group when the data of the two experiments were pooled.

[b] Statistical analysis of the antitumor experiments was done using a two-tailed Student *t* test. For all cases, results were regarded significant if *p* values were ≤0.05. (*p*) 1 and (*p*) 2 represent the *p* obtained when mean tumor volumes of the group injected with anti-HER2/*neu* IgG3-(GM-CSF) were compared with PBS and anti-HER2/*neu* IgG3 controls, respectively.

using anti-HER2/*neu* IgG3-(GM-CSF) may lead to enhanced macrophage activation at the site of the tumor; in murine models, activated macrophages given locally and i.v. inhibit tumor growth and decrease metastatic development (54).

Systemic clearance of anti-HER2/*neu* IgG3-(GM-CSF) is rapid compared with anti-HER2/*neu* IgG3. This is consistent with observations with other Ab cytokine fusion proteins (55), demonstrating a dominant role for the attached cytokine in determining the pharmacokinetics of the fusion proteins. We believe that the rapid clearance of the Ab fusion protein is through the GM-CSF receptors on normal cells (35) such as splenic T cells, B cells, and macrophages (56). In fact, our biodistribution studies showed that anti-HER2/*neu* IgG3-(GM-CSF) is mainly localized in the spleen consistent with earlier reports for the site targeted by murine GM-CSF (57). Interestingly, we found a dose-dependent rate of clearance with rapid clearance ($t_{1/2}$ = 2 h) seen when 0.5 μg was injected and slower clearance ($t_{1/2}$ = 10–12 h) when 20 μg was injected. Possibly, the higher doses saturated the available GM-CSF receptors. It has yet to be determined in patients whether the kinetics of clearance of anti-HER2/*neu* IgG3-(GM-CSF) will depend on the dose administered, although in a clinical study using nonglycosylated human GM-CSF injected i.v., no clear relationship between dose and half-life was observed (58). Despite its rapid clearance, anti-HER2/*neu* IgG3-(GM-CSF) retains the capacity to effectively target the tumor. In fact, the rapid clearance may be beneficial in clinical applications in which potentially injurious cytokine exposure to normal tissues should be minimized.

A half-life of ~30 h has been reported for the chCLL-1/GM-CSF fusion protein injected i.p. (53). The difference in clearance rates between anti-HER2/*neu* IgG3-(GM-CSF) and chCLL-1/GM-CSF may be explained by the use of different doses, by the route of injection (i.v. and i.p. respectively) and/or by the nature of the GM-CSF which were murine and human, respectively. Murine GM-CSF has considerably higher affinity for the murine GM-CSF receptor than does human GM-CSF (59), which may lead to more rapid clearance. A GM-CSF fusion protein specific for the murine transferrin receptor had a half-life of ~1.8 h (60). In this case, it is likely that the Ab fusion proteins were rapidly cleared by the ubiquitous transferrin receptor (61).

We have found that treatment with anti-HER2/*neu* IgG3-(GM-CSF) causes a significant retardation in the growth of s.c. CT26-HER2/*neu* tumors under conditions in which anti-HER2/*neu* IgG3 failed to confer protection. Our data are consistent with earlier experiments in which ch17217-(murine GM-CSF) specific for the murine transferrin receptor suppressed the development of pulmonary metastasis in five of eight immunocompetent mice injected with CT26. However, the control of Ab alone (ch17217) was not included in these earlier studies, making it impossible to distinguish the role of the Ab from that of GM-CSF (60). In those studies as well as our own, the control of Ab plus GM-CSF is also absent. Unfortunately, we did not have enough free GM-CSF available to include it as a control. Nevertheless, ours is the first report showing that an antitumor Ab-(GM-CSF) fusion protein shows a significant antitumor activity under conditions in which the Ab alone (anti-HER2/*neu* IgG3) fails to confer protection.

Several factors could explain our failure to obtain complete tumor remission. The dose, route, and schedule of treatment (daily i.v. injection of 20 μg for 5 days) may not be the optimal and/or

Table II. *Murine anti-human HER2/*neu *and anti-human IgG3 titers*[a]

Mouse	Anti-HER2/*neu* Titers			Anti-Human IgG Titers		
	PBS	IgG3	IgG3-(GM-CSF)	PBS	IgG3	IgG3-(GM-CSF)
1	150	150	12,150	N/A[b]	450	4,050
2	12,150	450	4,050	N/A	450	36,450
3	450	450	1,350	N/A	150	4,050
4	450	450	1,350	N/A	150	4,050
5	1,350	450	4,050	N/A	450	12,150
6	450	450	450	N/A	150	4,050
7	50	150	4,050	N/A	150	1,350
8	450	450	1,350	N/A	150	4,050

[a] Groups of eight mice injected s.c. with 10^6 CT26-HER2/*neu* cells were treated beginning the next day with five daily i.v. injections of 0.25 ml PBS containing 20 μg anti-HER2/*neu* IgG3-(GM-CSF), the equivalent molar amount of anti-HER2/*neu* IgG3, or nothing. Mice were bled 15 days after the injection of the tumor cells, and the sera were analyzed by a titration ELISA using plates coated with the ECD^{HER2} or human IgG3. The presence of Abs was detected using AP-labeled anti-mouse IgG. Values represent the average of duplicate dilutions of serum required to yield an optical density of 0.1 (410 nm) after 1 h of incubation.

[b] N/A, Not applicable.

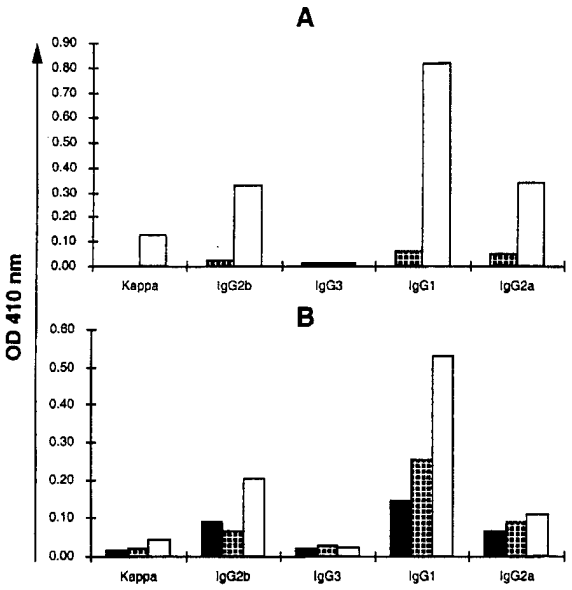

FIGURE 10. Isotype profile of Abs specific for HER2/*neu* and human IgG3. Pooled sera from mice treated with PBS (■), anti-HER2/*neu* IgG3 (▩), or anti-HER2/*neu* IgG3-(GM-CSF) (□) were analyzed by ELISA for Abs of different isotypes recognizing either anti-HER2/*neu* IgG3 (*A*) or ECD^{HER2} (*B*).

the tumor model may not be ideal for this particular study. In addition, we found that treatment with anti-HER2/*neu* IgG3-(GM-CSF) increases the endogenous humoral immune response against the human HER2/*neu* (39). Because we have evidence that endogenous Abs may inhibit the binding of recombinant anti-HER2/*neu* IgG3 to the tumor cells (39), this enhanced Ab response in anti-HER2/*neu* IgG3-(GM-CSF)-treated mice may further interfere with the binding of the anti-HER2/*neu* IgG3-(GM-CSF) to the cancer cells resulting in less effective antitumor activity. However, this may be a limitation only in the studies using murine tumors in which the expression of HER2/*neu* is not related to cell survival. In patients, the ability of anti-HER2/*neu* IgG3-(GM-CSF) to elicit a strong humoral immune response may be advantageous because Abs targeting HER2/*neu* on human tumors appear to directly inhibit their growth (15). Therefore, increasing the immune response using cytokines such as GM-CSF may facilitate tumor eradication. In fact, immunization using GM-CSF fused to the Ig expressed by a lymphoma can cause regression of the lymphoma in mice (62). The dramatically increased Ab response to the TAA HER2/*neu* is consistent with effective tumor targeting by anti-HER2/*neu* IgG3-(GM-CSF).

The isotype of the humoral immune response against human IgG and human HER2/*neu* suggests that anti-HER2/*neu* IgG3-(GM-CSF) has the ability to enhance both Th1 (T cell-directed) and Th2 (B cell-directed) immune responses. However, we do not know the effector mechanism responsible for the antitumor activity of anti-HER2/*neu* IgG3-(GM-CSF) observed in animals bearing CT26-HER2/*neu* tumors. Although ADCC mediated by effector cells such as macrophages, eosinophils, and NK cells is a possibility, $CD8^+$ (27) and $CD4^+$ (27, 30) cells may also play a role in that they have been shown to be necessary for protection against tumor cell challenge in mice vaccinated with irradiated GM-CSF-secreting tumor cells.

In conclusion, our results suggest that an anti-HER2/*neu* IgG3-(GM-CSF) fusion protein containing human GM-CSF may be effective in patients with tumors overexpressing HER2/*neu*. The combination of an anti-HER2/*neu* Ab with GM-CSF yields a protein with the potential to eradicate tumor cells by a number of mechanisms including the down-regulation of HER2/*neu* expression, ADCC, and the stimulation of a strong antitumor immune response through the immunostimulatory activity of GM-CSF. In addition, the anti-HER2/*neu* IgG3-(GM-CSF) fusion protein may be effective against tumor cells that express a truncated form of ECD^{HER2} lacking the receptor function rendering them particularly resistant to anti-HER2/*neu* Ab therapy (14). Because of the ability of GM-CSF to elicit an immune response to associated Ags, it is also possible that association of anti-HER2/*neu* IgG3-(GM-CSF) with soluble ECD^{HER2} shed by tumor cells will enhance the antitumor immune response.

Finally, we would like to stress that anti-HER2/*neu* IgG3-(GM-CSF) would not be a replacement for Herceptin but instead would provide an alternative therapy to be used in combination with the Ab or other anticancer approaches. These approaches might include chemotherapy or other anti-HER2/*neu* Ab fusion proteins such as anti-HER2/*neu* with the costimulator B7.1 (41) or the cytokine IL-12 (47). The availability of more than one Ab fusion protein will allow us to explore potential synergistic effects that may be obtained from manipulating the immune response.

Acknowledgments

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Appendix 2

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ORIGINAL ARTICLE

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A murine B cell lymphoma expressing human HER2/*neu* undergoes spontaneous tumor regression and elicits antitumor immunity

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Abstract In the present study we describe a novel murine tumor model in which the highly malignant murine B cell lymphoma 38C13 has been transduced with the cDNA encoding human tumor-associated antigen HER2/*neu*. This new cell line (38C13-HER2/*neu*) showed stable surface expression but not secretion of human HER2/*neu*. It also maintained expression of the idiotype (Id) of the surface immunoglobulin of 38C13, which serves as another tumor-associated antigen. Surprisingly, spontaneous tumor regression was observed following s.c. but not i.v. injection of 38C13-HER2/*neu* cells in immunocompetent syngeneic mice. Regression was more frequently observed with larger tumor cell challenges and was mediated through immunological mechanisms because it was not observed in syngeneic immunodeficient mice. Mice that showed complete tumor regression were immune to challenge with the parental cell line 38C13 and V1, a variant of 38C13 that does not express the Id. Immunity could be transferred with sera, suggesting that an antibody response mediated rejection and immunity. Continuously growing s.c. tumors as well as metastatic tumors obtained after the i.v. injection of 38C13-HER2/*neu* maintained expression of human HER2/*neu*, which can serve as a target for active immunotherapy. As spontaneous tumor regression has not been observed in other human murine models expressing human HER2/*neu*, our results illustrate the enormous differences that can exist among different murine tumors expressing the same antigen. The present model provides a useful tool for the study of the mechanisms of protective immunity to B cell lymphoma and for the evaluation of different therapeutic approaches based on the stimulation or suppression of the immune response.

Key words Tumor model · HER2/*neu* · Xenogenization · Immunotherapy · Lymphoma

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Introduction

The *HER2/neu* proto-oncogene (also known as c-*erbB*-2) encodes a 185-kDa transmembrane glycoprotein receptor that has partial homology with the epidermal growth factor receptor and shares with that receptor intrinsic tyrosine kinase activity. It consists of three domains: a cysteine-rich extracellular domain, a transmembrane domain and a short cytoplasmic domain [1, 12, 39]. HER2/*neu* is expressed at low levels on some normal cells; however, markedly increased expression has been observed in many human breast, gastrointestinal, lung and ovarian cancers [16, 35–37, 42]. The elevated levels of the HER2/*neu* protein in malignancies and the extracellular accessibility of this molecule make it an excellent candidate for tumor-specific therapeutic agents. In fact, treatment of patients with advanced breast cancer using the anti-HER2/*neu* antibody, trastuzumab (Herceptin, Genentech, San Francisco, Calif.), leads to an objective response in a subset of patients with tumors overexpressing the HER2/*neu* oncoprotein [2, 3, 45]. These results justify recent enthusiasm for continued efforts to refine existing approaches and to develop new strategies that target HER2/*neu*.

We have developed a family of anti-(human HER2/*neu*) antibody fusion proteins containing immunostim-

ulatory molecules such as the such as the cytokine interleukin-12 (IL-12) [29], costimulatory molecules such as B7.1 [10] or chemokines such as RANTES [11]. To evaluate the immunological efficacy of these proteins, it is critical that tumors expressing the target antigen can grow in immunologically competent mice. To produce murine tumors expressing human HER2/*neu*, we transduced the murine colon adenocarcinoma cell lines CT26 and MC38 and the murine T cell lymphoma EL4 with the cDNA encoding the human HER2/*neu*. We showed that those cells were able to grow in immunocompentent mice while maintaining the expression of human HER2/*neu* [31] and such models are now being used for preclinical evaluation of the efficacy of anti-HER2/*neu* antibody fusion proteins [29] (M.L. Penichet et al. unpublished results).

To further expand our repertoire of human-HER2/*neu*-expressing murine tumors, we have developed a new model using the highly malignant murine B cell lymphoma 38C13. Although overexpression of HER2/*neu* has been mainly associated with breast, gastrointestinal, lung and ovarian cancers [16, 35–37, 42], it has also been described for B cell lymphoma [17, 18]. Thus, 38C13 expressing human HER2/*neu* may be used to evaluate the efficacy of antibody fusion proteins against B cell lymphoma. If these proteins are effective they can be further evaluated in clinical trials targeting lymphomas expressing HER2/*neu* or they can be modified to target other tumor-associated antigens (TAA) found on B cell lymphomas. In addition, since the Id of the surface immunoglobulin of 38C13 has been previously used to target anti-Id antibody fusion proteins [25, 30], the 38C13-HER2/*neu* model will allow us to test the potential synergistic effect of different antibody fusion proteins targeting two different TAA: the Id and HER2/*neu*. Moreover, the availability of different cell lines expressing the same antigen allows us to test the efficacy of HER2/*neu*-targeted approaches in a variety of cell lines and/or mouse strains and it is well known that different responses to the same anticancer therapy are exhibited by different tumors [30].

In this report, we describe the transduction of 38C13 with a retroviral construct containing the full-length cDNA encoding the human HER2/*neu* gene. These cells (38C13-HER2/*neu*) show stable expression of human HER2/*neu* on their surface while maintaining expression of the surface immunoglobulin. The biological properties of this transduced cell line were analyzed after transplantation into immunologically intact syngeneic mice. Parameters that were investigated include tumor growth rate and phenotype, ability to produce metastases, expression of HER2/*neu*, antigen shedding and the anti-(human HER2/*neu*) response of the host. Contrary to our expectations, spontaneous tumor regression after temporary growth was observed following s.c. injection of tumor cells, and this regression was more frequently observed the greater the number of cells used to elicit tumor growth; however, regression was not observed following i.v. injection of tumor cells.

Materials and methods

Cells

38C13 is a C3H/HeN murine B cell lymphoma expressing a surface $\mu\kappa$ antibody (Id) that arose in a carcinogen(7,12-dimethylbenz [*a*]anthracene)-treated mouse [4, 5]. V1 is an Id-negative variant derived from the original 38C13 tumor [38]. Both cell lines were kindly provided by Drs. Ronald and Shoshana Levy (Stanford University, Stanford, Calif.). The parental and transduced cells were maintained in Iscove's modification of Dulbecco's medium (Irvine Scientific Inc., Irvine, Calif.) containing 10% calf serum supplemented with iron (Atlanta Biologicals, Norcross, Ga.) at 37 °C with 5% CO_2.

Mice

Female immunocompetent C3H/HeN mice and Rag2 double-knockout mice lacking mature T and B cell lymphocytes with the C3H/HeN background, between 6–8 weeks of age, obtained from Taconic Farms Inc. (Germantown, N.Y.) were used. The mice received food and water ad libitum. Artificial light was provided under a 12 h/12 h light/dark cycle. The temperature of the facility was 20 °C with 10–15 air exchanges/h. All experiments were performed according to National Institutes of Health (NIH) (Bethesda, Md.) *Guide for the care and use of laboratory animals.*

Retroviral expression vector, transduction and screening

The cDNA for HER2/*neu* cloned in retroviral vector based on a Moloney murine leukemia virus (MoMuLV), including the neomycin-resistance gene (*neo*) under the control of the SV40 promoter, was used for transduction of 38C13. This vector was previously used to derive other human HER2/*neu*-expressing murine cell lines [31]. Cells were selected with geneticin (Sigma Chemical, St. Louis, Mo.). HER2/*neu* expression on the surface of transduced cells was detected with an immunofluorescence assay. Samples of 10^6 cells were incubated with 1 µg recombinant anti-HER2/*neu* antibody for 2 h at 4 °C. Recombinant anti-Id and recombinant anti-DNS (DNS is the hapten *N,N* dimethyl-1-aminonaphthalene-5-sulfonyl chloride, also known as dansyl) antibodies were used as positive and negative isotype-matched controls respectively. All three recombinant antibodies were developed in our laboratory and contained human κ and $\gamma 3$ constant regions. Cells were washed and, following incubation for 2 h at 4 °C with biotinylated goat anti-(human IgG) (Pharmingen, San Diego, Calif.), were washed and incubated for 30 min with phycoerythrin-labeled streptavidin (Pharmingen, San Diego, CA). Analysis was performed with a FACScan flow cytometer (Becton-Dickinson, Mountain View, Calif.) equipped with a blue laser excitation of 15 mW at 488 nm.

Determination of the presence of extracellular domain of HER2/*neu* antigen (ECD^{HER2}) in cell culture supernatant

ECD^{HER2} was detected by enzyme-linked immunosorbent assay (ELISA). Ninety-six well microtiter plates were coated with 50 µl recombinant human anti-HER2/*neu* IgG3 (developed in our laboratory) at a concentration of 1 µg/ml. The plates were blocked with 3% bovine serum albumin (BSA) in phosphate-buffered saline (PBS) and dilutions of cell culture supernatant or ECD^{HER2} (kindly provided by Dr. James D. Marks, UCSF; San Francisco, Calif.) in PBS containing 1% BSA were added to the wells and incubated overnight at 4 °C. The wells were then washed with PBS and incubated for 2 h at room temperature with the anti-HER2/*neu* mAb Neu 9G6 (Santa Cruz Biotechnology, Inc., Santa Cruz, Calif.). After washing with PBS, alk-aline-phosphatase-labeled goat anti-(mouse IgG) (Sigma Chemical, St. Louis, Mo.) was added and the

plates were incubated for 1 h at 37 °C. After washing, disodium *p*-nitrophenyl phosphate dissolved in diethanolamine buffer (Sigma Chemical, St. Louis, Mo.) was added to the wells for 1 h and plates were read at 410 nm.

s.c. and i.v. transplantation of tumor cell lines

Mice were injected s.c. in the right flank with 38C13-HER2/*neu* in 0.15 ml Hanks's balanced salt solution (HBSS) (Gibco BRL, Grand Island, N.Y.). In order to compare the s.c. growth of the transduced cell lines with that of their respective parental cell line, additional groups were injected with the same dose of 38C13. Tumor growth was measured three times a week with a caliper and the length of survival recorded. Mice were injected i.v. with 38C13-HER2/*neu* or with the corresponding dose of 38C13 in 0.3 ml HBSS (Gibco BRL, Grand Island, N.Y.) via the lateral tail vein, and the length of survival was recorded.

Histological study

Mice were injected s.c. with 38C13-HER2/*neu* or 38C13 and tumor growth monitored described above. Mice were humanely killed 24 h after tumor regression started and a histological study of their respective tumors was carried out on paraformaldehyde-fixed paraffin-embedded tissue samples. Sections of 6 μm were stained with hematoxylin/eosin.

Passive transfer of sera and splenocytes

A pool of sera and splenocytes was prepared from mice that had shown tumor regression and survived 60 days without new evidence of tumor following injection with 38C13-HER2/*neu*. According to previous reports, mice inoculated with 38C13 that are free of tumor 60 days after the injection are, in fact, cured of lymphoma [6, 7, 30]. A splenocyte suspension was prepared by mincing and compressing freshly resected spleens between two slides on a petri dish in the presence of HBSS. The splenic capsule was discarded and the cell suspension was transferred to a polystyrene tube. After washing twice with HBSS, the cells were counted using crystal violet staining (Sigma Chemical, St. Louis, Mo.). To transfer immunity using splenocytes, 5×10^7 cells in 0.3 ml HBSS were injected into the tail vein of naïve syngeneic recipient mice. All mice receiving splenocytes were injected i.p. with 100 IU heparin (Sigma Chemical, St. Louis, Mo.) 30 min before the transfer of the splenocytes. To transfer immunity using sera, 0.3 ml pooled sera was injected into the tail vein of naïve syngeneic recipient mice. One day later, mice were challenged s.c. with a lethal dose (10^4) of the parental tumor 38C13 cells. Tumor incidence and survival were monitored. Naïve syngeneic recipient mice that did not receive splenocytes or sera, or that received splenocytes or sera from naïve mice of a similar age to the long-term survivors, were used as controls.

Determination of murine anti-HER2/*neu* and anti-Id antibodies

The presence of antibodies to human HER2/*neu* or to murine Id in mice sera was determined by ELISA using 96-well microtiter plates coated with 50 μl (at a concentration of 1 μg/ml) of ECD^{HER2} or with Id obtained from concentrated supernatant of the hybridoma A1–2, which secretes high levels of soluble 38C13 Id [28]. The plates were blocked with 3% BSA in PBS and dilutions of serum in PBS containing 1% BSA were added to the wells and incubated overnight at 4 °C. The wells were then washed with PBS, alkaline-phosphatase-conjugated goat anti-(mouse IgG) (Sigma Chemical, St. Louis, Mo.) was added, and the plates were processed as described above. As a negative control for determining anti-HER2/*neu* titers, we used sera from mice of the same age bearing tumors of non-transduced 38C13. As a negative control for determining anti-Id titers we used sera from naïve mice of the same age. All ELISA for comparison of titers between 38C13 and 38C13-HER2/*neu* were carried out simultaneously in duplicate and using an internal positive control curve for each plate.

Detection of HER2/*neu* and Id expression in tumors by flow cytometry

Single-cell suspensions from 38C13-HER2/*neu* and 38C13 were prepared by mincing and pipetting freshly isolated tumors in cold medium. The detection of Id and HER2/*neu* surface expression on a fresh single-cell suspension, as well as on the same cells kept for 1 week in tissue culture, was done by flow cytometry, as described above.

Statistical analysis

Statistical analysis of the differential findings between experimental groups of mice was done using the nonparametric Wilcoxon-Mann-Whitney rank-sum test. This allows us to include both dead mice as well as the long-term survivors in the analysis.

Results

In vitro human HER2/*neu* expression in transduced cells

The murine tumor cell line 38C13 was transduced with the retroviral vector containing the HER2/*neu* cDNA under the control of the MoMuLV enhancer/promoter (38C13-HER2/*neu*). A stable pool of cells selected in geneticin was tested for surface expression of human HER2/*neu* by flow cytometry. Human HER2/*neu* expression was detected on the surface of the transduced cell line (Fig. 1B). Incubation of the anti-HER2/*neu* IgG3 with 38C13-HER2/*neu* in the presence of excess soluble ECD^{HER2} abrogated binding (data not shown), confirming the specificity of the recombinant anti-HER2/*neu* IgG3 used in this assay. The expression of the Id of the μκ surface immunoglobulin was also detected by flow cytometry (Fig. 1C). The level of Id expression in 38C13-HER2/*neu* was similar to the level found on the parental cell line (Fig. 1F).

The above results confirmed the surface expression of human HER2/*neu*. Secretion of the ECD^{HER2} has been reported for some HER2/*neu*-expressing tumors [24, 32]. To address this issue, we quantified the amount of ECD^{HER2} present in the culture supernatant of 38C13-HER2/*neu* cells grown at 10^6/ml and incubated for 24 h. Although the ELISA assay used can detect more than 2 ng/ml soluble recombinant ECD^{HER2}, we did not detect the presence of the ECD^{HER2} in culture supernatants of cells carried in tissue culture or isolated from tumors and expanded in vitro (data not shown).

s.c. tumor growth characteristics

The growth kinetics in the s.c. space of normal syngeneic mice of 38C13-HER2/*neu* was compared to that of the parental cell line 38C13. Doses of 10^3 and 10^4 38C13 cells injected s.c. have been shown to yield tumors in 100% of mice [6, 7, 20, 25, 28, 30]. For this reason, we

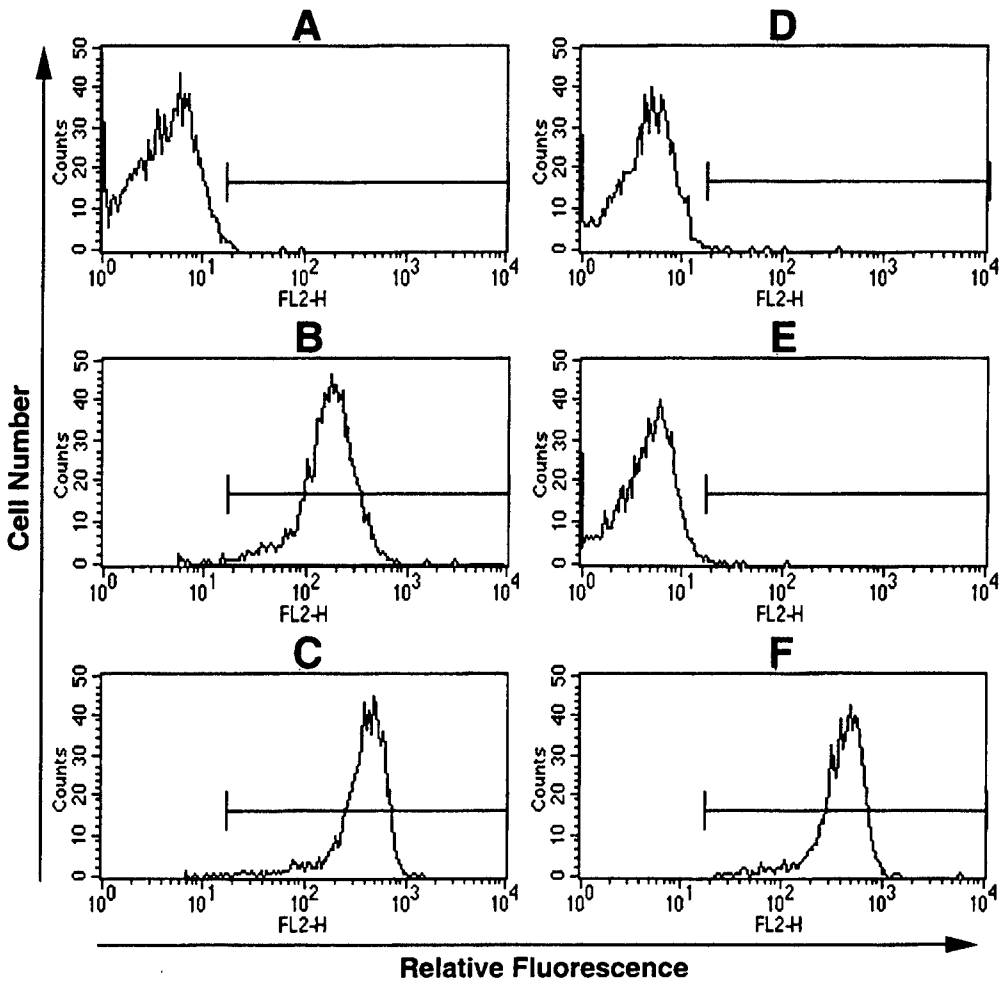

Fig. 1A–F Analysis by flow cytometry of the surface expression of human HER2/*neu* and Id by 38C13-HER2/*neu* (**A–C**) or 38C13 (**D–F**). Cells were stained with anti-DNS human IgG3 (**A**, **D**), anti-HER2/*neu* human IgG3 (**B**, **E**) or anti-Id human IgG3 (**C**, **F**), followed by biotinylated goat anti-(human IgG) and phycoerythrin-labeled streptavidin

injected groups of 5 mice in the right flank with 10^3, 10^4, 10^5 or 10^6 38C13-HER2/*neu* or 38C13 cells. 100% of the mice developed tumors with a similar time of tumor onset for each dose of 38C13-HER2/*neu* and 38C13 (Fig. 2). As expected, higher doses of both cell lines resulted in shorter latency. However, when the tumors reached a size of approximately 1 cm in diameter, some of the 38C13-HER2/*neu* tumors showed spontaneous regression. This phenomenon appeared to be dose-related as complete regression was more frequently observed with larger tumor cell challenges: 1/5 for 10^3, (20%), 4/5 for 10^4 (80%), 4/5 for 10^5 (80%) and 5/5 for 10^6 (100%) (Fig. 3, Table 1). All mice showing complete tumor regression became long-term survivors. As expected, all mice injected with 10^3, 10^4 or 10^5 38C13 cells developed progressive tumors and died (Figs. 2, 3). Surprisingly, 1 of the 5 mice injected with 10^6 38C13 cells also showed spontaneous tumor rejection and became a long-term survivor. The survival of mice inoculated with 10^4, 10^5 and 10^6 38C13-HER2/*neu* cells was significantly better than that of mice injected with 38C13 ($P < 0.05$). Although the survival of mice injected with 10^3 cells was not significantly different whether 38C13-HER2/*neu* or 38C13 was used, 1 of 5 mice injected with 38C13-HER2/*neu* became a long-term survivor, while all of the mice injected with 38C13 cells died. Post-mortem studies of mice injected with s.c. 38C13 or 38C13-HER2/*neu* tumors revealed the presence of metastatic tumors in lymph nodes throughout the body (data not shown).

A repetition of this experiment using 10 mice/group for injections of 10^3, 10^4 or 10^5 cells and 20 mice/group for injection of 10^6 cells gave similar results (Table 1). Larger tumor cell challenges of 38C13-HER2/*neu* were associated with more frequent complete spontaneous tumor regression. As expected, the injection of 10^3, 10^4 or 10^5 38C13 cells resulted in progressively growing tumors in 100% of mice, but following the injection of 10^6 38C13, spontaneous tumor regression was seen in 2 of 20 (10%) mice.

Two months after the s.c. tumors regressed, long-term survivors were challenged with the 10^4 38C13 cells in the left flank. At this tumor cell dose all naïve mice of the same age showed progressive tumor growth and died. In contrast, 100% of the long-term survivors previously injected with 10^4, 10^5 or 10^6 38C13-HER2/*neu* remained free of tumor (Table 2). Similar results were observed with 2 of the 3 (67%) mice previously injected with 10^3 38C13-HER2/*neu*, suggesting that this lower dose is not only associated with less frequent spontaneous tumor regression but also with weaker antitumor

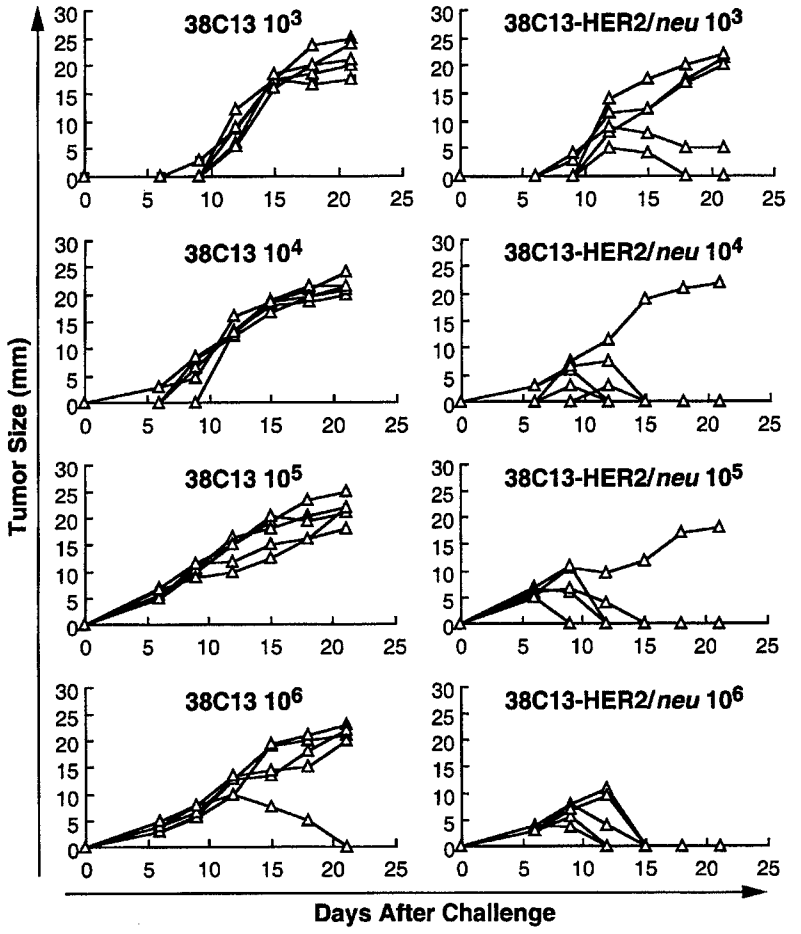

Fig. 2 Kinetics of tumor growth. Groups of 5 mice were injected in the right flank with 10^3,10^4, 10^5 or 10^6 cells from either 38C13 or 38C13-HER2/*neu*. Tumor growth was monitored and measurements were recorded three times per week with a caliper

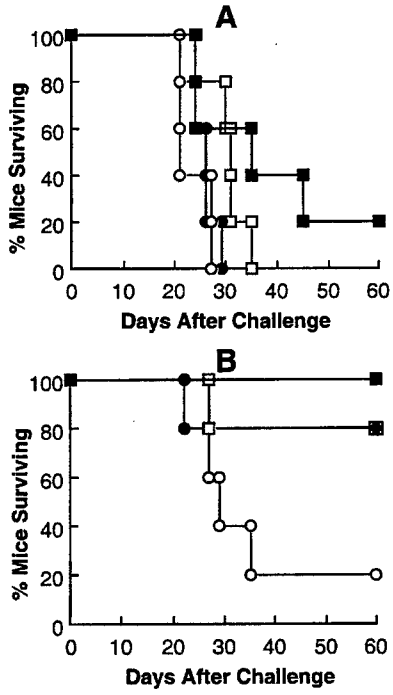

Fig. 3A, B Survival of C3H/HeN mice (5/group) inoculated s.c. with 10^3 (○), 10^4 (●), 10^5 (□), or 10^6 (■) 38C13 (**A**) or 38C13-HER2/*neu* (**B**)

Table 1 Survival of C3H/HeN mice challenged with different doses of 38C13 or 38C13-HER2/*neu*

Tumor injection	Number of mice	Number of survivors
Experiment 1		
10^3 38C13-HER2/*neu*	5	1
10^4 38C13-HER2/*neu*	5	4
10^5 38C13-HER2/*neu*	5	4
10^6 38C13-HER2/*neu*	5	5
10^3 38C13	5	0
10^4 38C13	5	0
10^5 38C13	5	0
10^6 38C13	5	1
Experiment 2		
10^3 38C13-HER2/*neu*	10	2
10^4 38C13-HER2/*neu*	10	4
10^5 38C13-HER2/*neu*	10	4
10^6 38C13-HER2/*neu*	20	16
10^3 38C13	10	0
10^4 38C13	10	0
10^5 38C13	10	0
10^6 38C13	20	2

immunity. When long-term survivors previously injected with 10^6 38C13-HER2/*neu* were challenged with a lethal dose of 10^4 V1 cells, an Id-negative variant derived from the original 38C13, 100% of mice remained free of

Table 2 Survival of mice challenged with 38C13 or V1 cells. C3H/HeN mice that showed complete tumor rejection following injection with 38C13-HER2/*neu* were challenged with a lethal dose (10^4) of 38C13 or V1, an Id-negative variant derived from 38C13. The challenge was made 2 months after the primary tumor had completely regressed. As controls, the same number of naïve C3H/HeN mice of similar age were injected with 38C13 or V1

Previous dose of injection	Challenge	Number of mice	Number of survivors
10^3	38C13 (10^4)	3	2
10^4	38C13 (10^4)	5	5
10^5	38C13 (10^4)	7	7
10^6	38C13 (10^4)	20	20
None (controls)	38C13 (10^4)	35	0
10^6	V1 (10^4)	8	8
None (controls)	V1 (10^4)	8	0

tumor while all naïve mice of the same age showed progressive tumor growth and died (Table 2).

To compare the histology of regressing and non-regressing tumors, groups of 5 mice were injected s.c. in the right flank with 10^6 38C13-HER2/*neu* or 38C13. As expected, all of the mice initially developed tumors (data not shown). By day 11 after the injection of the cells, regression had begun in all of the mice injected with 10^6 38C13-HER2/*neu*. No tumor regression was observed in mice injected with 10^6 38C13. Figure 4 shows histological sections stained with hematoxylin/eosin of tumors obtained from mice 12 days following injection. The 38C13 tumor is a highly cellular tumor that infiltrates the subcutaneous space and part of the muscular coat (Fig. 4A). Examination of regressing 38C13-HER2/*neu* tumor was characterized by intense eosinophilia, cell shrinkage, loss of structure and fragmentation, all classic images of necrosis [26] (Fig. 4B). Similar results were observed in all mice injected with 38C13-HER2/*neu* cells (data not shown). All mice injected with 38C13 had highly cellular tumors similar to the tumor depicted in Fig. 4A.

To test whether tumor regression was immunologically mediated, groups of 8 syngeneic Rag2 double-knockout mice, which lack mature T and B lymphocytes, received s.c. injections of 10^6 38C13 or 38C13-HER2/*neu* in the right flank. In contrast to what had been observed with immunocompetent mice, both cell lines showed similar tumor growth and no tumor regression was observed in mice injected with 38C13 or 38C13-HER2/*neu* (data not shown).

Passive transfer of sera and splenocytes from long-term survivors

To determine if humoral or cellular immunity was responsible for the protection observed in mice showing spontaneous tumor regression, either serum or splenocytes from mice that had previously rejected s.c. 38C13-HER2/*neu* tumors was transferred to naïve C3H/HeN mice 24 h prior to tumor challenge. Transfer of splenocytes or serum was found to confer significant protection to challenge 24 h later, with 10^4 38C13 cells causing either retardation or resistance to tumor growth (Fig. 5). Survival of mice receiving splenocytes or serum from immune mice was significally greater than that of the control group that did not receive treatment: $P < 0.001$ for mice receiving splenocytes and $P = 0.01$ for mice receiving serum. Mice receiving a similar amount of serum or splenocytes from naïve mice showed survival curves similar to those of mice that did not receive any treatment (data not shown).

i.v. tumor growth characteristics

The in vivo studies described above have been restricted to tumors growing in the s.c. space. However, as the route of injection can influence the growth potential of certain cell lines, we also investigated the growth of 38C13-HER2/*neu* after i.v. injection. Groups of 5 mice were injected i.v. with 10^3, 10^4, 10^5, or 10^6 38C13-HER2/*neu* or 38C13 cells. We found that, after injecting the cells i.v., all of the mice injected with 38C13-HER2/*neu* or 38C13 developed a disseminated malignant disease leading to death (Fig. 6). No long-term survivors were observed. Post-mortem studies revealed the presence of tumor metastases in the lymph nodes throughout the body in all mice. Similar results were obtained when this experiment was repeated (data not shown).

Determination of the anti-(human HER2/*neu*) and anti-Id response in mice bearing s.c. and i.v. tumors

To determine if anti-(human HER2/*neu*) or anti-Id antibodies were elicited in mice bearing s.c. tumors, groups of 5 mice inoculated s.c. with 10^4 38C13-HER2/*neu* or 38C13 tumor cells were bled every 3 days for 15 days following injection of the cells. Table 3 shows that anti-(human HER2/*neu*) antibodies were seen in all of the mice bearing HER2/*neu*-expressing tumors by day 12 with response detectable in 2/5 (40%) of the mice 6 days after challenge. The sera were also tested by ELISA for the presence of anti-Id (Table 3). We did not detect anti-Id in mice bearing the parental tumor 38C13. However, in mice bearing 38C13-HER2/*neu* tumors a detectable anti-Id response was observed in 3/5 (60%) by day 12. This anti-Id immune response was observed in 3 of the 4 mice showing tumor regression but not in the mouse with a continuously growing 38C13-HER2/*neu* tumor.

To compare the titers of anti-(human HER2/*neu*) or anti-Id antibodies elicited in mice bearing s.c. tumors induced by different doses of cells, groups of 5 mice (randomly selected) injected in the right flank with 10^3, 10^4, 10^5 or 10^6 38C13-HER2/*neu* or 38C13 cells were bled 12 days after the injection of the cells and the sera analyzed for the presence of anti-(human HER2/*neu*) or anti-Id antibodies (Table 4). No anti-human HER2/*neu* or anti-Id was detected in sera collected from mice injected with 10^3 38C13-HER2/*neu*. However, at higher doses of 38C13-HER2/*neu* cells, both anti-(human HER2/*neu*)

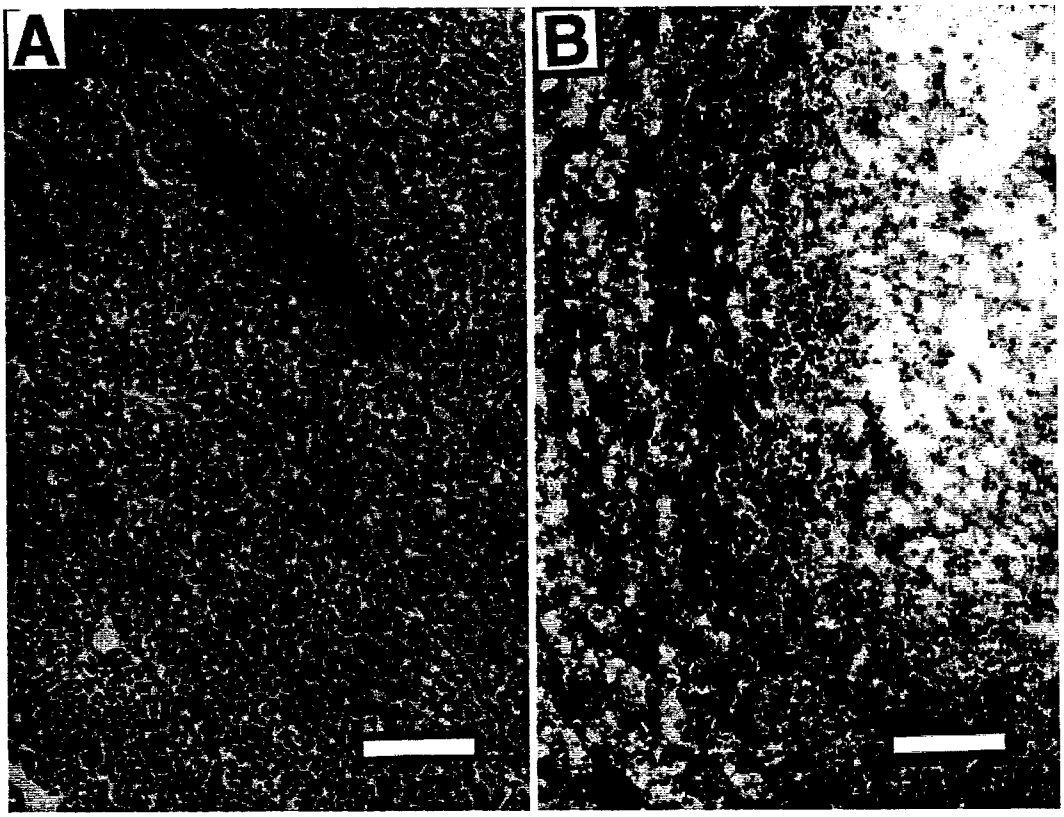

Fig. 4A, B Histological sections of a 12-day-old continuously growing 38C13 tumor (**A**) or a 12-day-old regressing 38C13-HER2/*neu* tumor (**B**). Both tumors were from C3H/HeN mice injected s.c. with 10^6 tumor cells. The histological study was carried out on paraformaldehyde-fixed, paraffin-embedded 6-μm sections stained with hematoxylin/eosin. *Bar* 100 μm

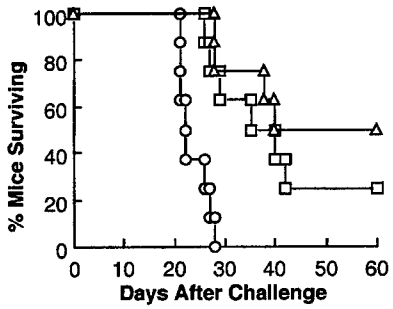

Fig. 5 Survival of C3H/HeN-mice challenged s.c. with a lethal dose of 10^4 38C13. One day before challenge, groups of 8 mice each received a single i.v. injection of 5×10^7 splenocytes from immune animals previously inoculated with 38C13-HER2/*neu* (△) or 0.3 ml serum from immune animals (□) or untreated animals (○)

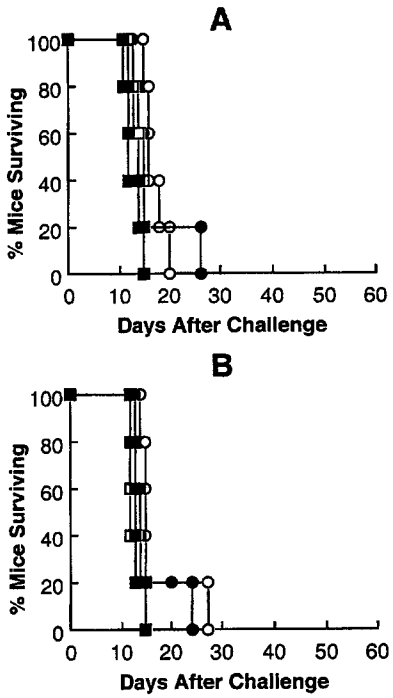

Fig. 6A, B Survival of C3H/HeN mice (5/group) inoculated i.v. with 10^3 (○), 10^4 (●), 10^5 (□), or 10^6 (■) 38C13 (**A**) or 38C13-HER2/*neu* (**B**)

and anti-Id antibodies were seen. Consistent with the results presented in Table 3, mice injected with 10^4 38C13 did not show an anti-Id response by day 12 while 3/5 of mice injected with 10^4 38C13-HER2/*neu* showed anti-Id titers. Inoculation with higher doses (10^5 or 10^6) of 38C13-HER2/*neu* resulted in higher anti-Id titers than when an equivalent dose of 38C13 was used. It is important to note that no direct correlation is seen between the magnitude of the anti-(human HER2/*neu*) response and the survival of mice, suggesting that the anti-(human HER2/*neu*) antibody response may not significantly contribute to survival. In contrast, the anti-Id response

Table 3 Kinetics of anti-(human HER2/*neu*) and anti-Id antibody response in mice bearing 38C13-HER2/*neu* or 38C13 tumors. Groups of 5 mice were injected in the right flank with 10^4 38C13-HER2/*neu* or 38C13 cells. Mice were bled every 3 days and the sera analyzed by a titration enzyme-linked immunosorbent assay (ELISA) using plates coated with the ECD^{HER2} or Id. The presence of antibodies was detected using alkaline-phosphatase-labeled anti-(mouse IgG). Values represent the average of duplicate dilutions of serum required to yield an absorbance of 0.1 (410 nm). Tumor growth was monitored and measurements were recorded three times per week with a caliper and was classified as progression (*P*) or regression (*R*)

Cells injected	Antibody tested	Mouse number	Antibody level after challenge					Tumor response
			3 days	6 days	9 days	12 days	15 days	
38C13-HER2/*neu*	Anti-(human HER2/*neu*)	1	0	50	150	50	4050	R
		2	0	0	50	450	1350	R
		3	0	0	0	1350	1350	R
		4	0	0	50	50	450	P
		5	0	50	150	1350	4050	R
	Anti-(mouse Id)	1	0	0	0	50	1350	R
		2	0	0	0	450	1350	R
		3	0	0	0	150	450	R
		4	0	0	0	0	0	P
		5	0	0	0	0	0	R
38C13	Anti-(mouse Id)	1	0	0	0	0	0	P
		2	0	0	0	0	0	P
		3	0	0	0	0	0	P
		4	0	0	0	0	0	P
		5	0	0	0	0	0	P

Table 4 Comparison of anti-(human HER2/*neu*) and anti-Id levels in mice bearing s.c. 38C13-HER2/*neu* or 38C13 tumors. Groups of 5 mice injected in the right flank with 10^3, 10^4,10^5 or 10^6 38C13-HER2/*neu* or 38C13 cells were bled 12 days after the injection of the cells and the sera analyzed by a titration ELISA using plates coated with the ECD^{HER2} or Id. The presence of antibodies was detected using alkaline-phosphatase-labeled anti-(mouse IgG). Values represent the average of duplicate dilutions of serum required to yield an absorbance of 0.1 (410 nm). Tumor growth was monitored and measurements were recorded three times per week with a caliper. Growth was classified as progression (*P*) or regression (*R*)

Cells injected	Antibody tested	Mouse number	Antibody level and tumor response after injection of:			
			10^3 cells	10^4 cells	10^5 cells	10^6 cells
38C13-HER2/*neu*	Anti-(human HER2/*neu*)	1	0 P	450 P	4050 P	450 R
		2	0 P	150 R	450 R	450 P
		3	0 R	50 P	1350 P	1350 R
		4	0 R	50 R	450 P	450 R
		5	0 P	450 R	450 P	450 R
	Anti-(mouse Id)	1	0 P	50 P	150 P	450 R
		2	0 P	150 R	450 R	150 P
		3	0 R	0 P	50 P	450 R
		4	0 R	50 R	150 P	1350 R
		5	0 P	0 R	0 P	450 R
38C13	Anti-(mouse Id)	1	0 P	0 P	150 P	50 P
		2	0 P	0 P	150 P	450 P
		3	0 P	0 P	0 P	150 P
		4	0 P	0 P	0 P	150 P
		5	0 P	0 P	50 P	150 P

may be associated with an effective antitumor response. For mice injected with 10^6 38C13-HER2/*neu* cells, the only mouse showing tumor progression exhibited the lowest anti-Id titer. In the group of mice injected with 10^5 38C13-HER2/*neu* cells, the only mouse showing tumor regression exhibited the highest anti-Id titer. In the group of mice injected with 10^4 cells, 1 of the 3 mice showing tumor regression exhibited the highest anti-Id titer while the other 2 mice exhibited titers similar (0 or 50) to those of mice showing tumor progression. No anti-Id was detected in mice injected with 10^3 38C13-HER2/*neu* even though 2 mice showed tumor regression. However, we should stress that, at day 12, tumor regression had not yet begun in mice injected with 10^3 or 10^4 cells, while mice injected with 10^5 or 10^6 cells already showed clear signs of regression or progression. Thus, the day 12 response may not accurately reflect the association between anti-Id titers and the antitumor response.

Table 4 also shows that no anti-Id was seen in sera collected from day-12 mice injected with 10^3 or 10^4 parental 38C13 cells. However, increasing the injection dose of 38C13 cells resulted in detectable titers of anti-Id antibodies in 3/5 (60%) mice challenged with 10^5 38C13 and in 5/5 (100%) of mice challenged with 10^6 38C13. These anti-(mouse Id) titers, however, are clearly lower than that those found in mice challenged with 10^4, 10^5, 10^6 38C13-HER2/*neu* and were not associated with tumor regression.

We also studied the anti-(human HER2/*neu*) and anti-Id response elicited by day 12 in mice injected with 10^3, 10^4, 10^5 or 10^6 38C13-HER2/*neu* i.v. (Table 5). Interestingly, anti-(human HER2/*neu*) titers are present in 4/5 mice injected with 10^3 38C13-HER2/*neu*; however, in contrast with what was seen in mice injected s.c., increasing the injection dose did not increase the anti-(human HER2/*neu*) titers. No anti-Id response was detected in any mice injected with 10^3, 10^4 or 10^5 38C13-HER2/*neu* cells and only a modest response was observed in 3/5 (60%) of mice injected with 10^6 38C13-HER2/*neu* cells.

In vivo tumor expression of HER2/*neu* and Id as detected by flow cytometry

Flow-cytometry analysis of freshly isolated cells from 15-day-old s.c. (Fig. 7A, B) or metastatic (Fig. 7C, D) 38C13-HER2/*neu* tumors growing in cervical lymph nodes from four different mice inoculated with 10^4 cells showed persistence of cell-surface expression of human HER2/*neu* in all tumors. However, the level of surface expression of human HER2/*neu* determined immediately following removal of tumor from the mice appears decreased compared with cells maintained in tissue culture. After 1 week in culture the level of human HER2/*neu* detected markedly increased (Fig. 7E–H) although, in some cases, not to the same level seen with cells maintained continuously in tissue culture. The level of surface expression of Id from the freshly isolated tumors described above appears to be identical to that of cells maintained in tissue culture (data not shown). Similar results have been observed with cells isolated from eight additional continuously growing s.c. 38C13-HER2/*neu* tumors dissected from different mice injected s.c. and from eight additional metastatic tumors growing in the lymph nodes of mice injected i.v. (data not shown). We have also confirmed that the level of HER2/*neu* and Id expression is similar among three different metastatic tumors growing in the same mouse and from four tumors from different mice injected i.v. (data not shown). The above results suggest that in vivo selection of variants lacking the expression of human HER2/*neu* or Id does not occur.

Discussion

The 38C13 B-cell lymphoma was successfully transduced with a retroviral construct containing the full-length cDNA encoding human HER2/*neu*. We found that the transduced cells show stable high-level surface expression of both TAA: human HER2/*neu* and Id. We also found no secretion of soluble HER2/*neu*. ECD^{HER2} is known to be released by some cancer cells that overexpress HER2/*neu* [24, 32, 47] and elevated ECD^{HER2} serum levels have been described in patients with breast cancer [21, 32]. The secretion of ECD^{HER2} has been reported to be a drawback for anti-HER2/*neu* therapy in humans. In fact, the dose of the rhu MAb HER2, now in clinical use, provides adequate serum concentrations in all patients except those with serum levels of tumor-shed ECD^{HER2} of 500 ng/ml or more [2, 3]. Tumor-shed Id has been described as a significant limitation for antibody-based therapeutic approaches targeting the Id expressed on lymphomas [28]. Mice bearing the 38C13 tumors accumulate only a small amount of antibodies bearing the Id during the first week following injection

Table 5 Comparison of anti-(human HER2/*neu*) and anti-Id levels in mice injected i.v. with 38C13-HER2/*neu* cells. Groups of 5 mice injected by the vein tail with 10^3, 10^4,10^5 or 10^6 38C13-HER2/*neu* cells were bled 12 days after the injection of the cells and the sera analyzed by a titration ELISA using plates coated with the ECD^{HER2} or Id. The presence of antibodies was detected using alkaline-phosphatase-labeled anti-(mouse IgG). Values represent the average of duplicate dilutions of serum required to yield an absorbance of 0.1 (410 nm)

Cells injected	Antibody tested	Mouse number	Antibody level after injection of:			
			10^3 cells	10^4 cells	10^5 cells	10^6 cells
38C13-HER2/*neu*	Anti-(human HER2/*neu*)	1	0	150	50	0
		2	50	Dead	Dead	150
		3	150	150	50	50
		4	50	Dead	150	50
		5	450	50	50	150
	Anti-(mouse Id)	1	0	0	0	50
		2	0	Dead	Dead	0
		3	0	0	0	0
		4	0	Dead	0	150
		5	0	0	0	50

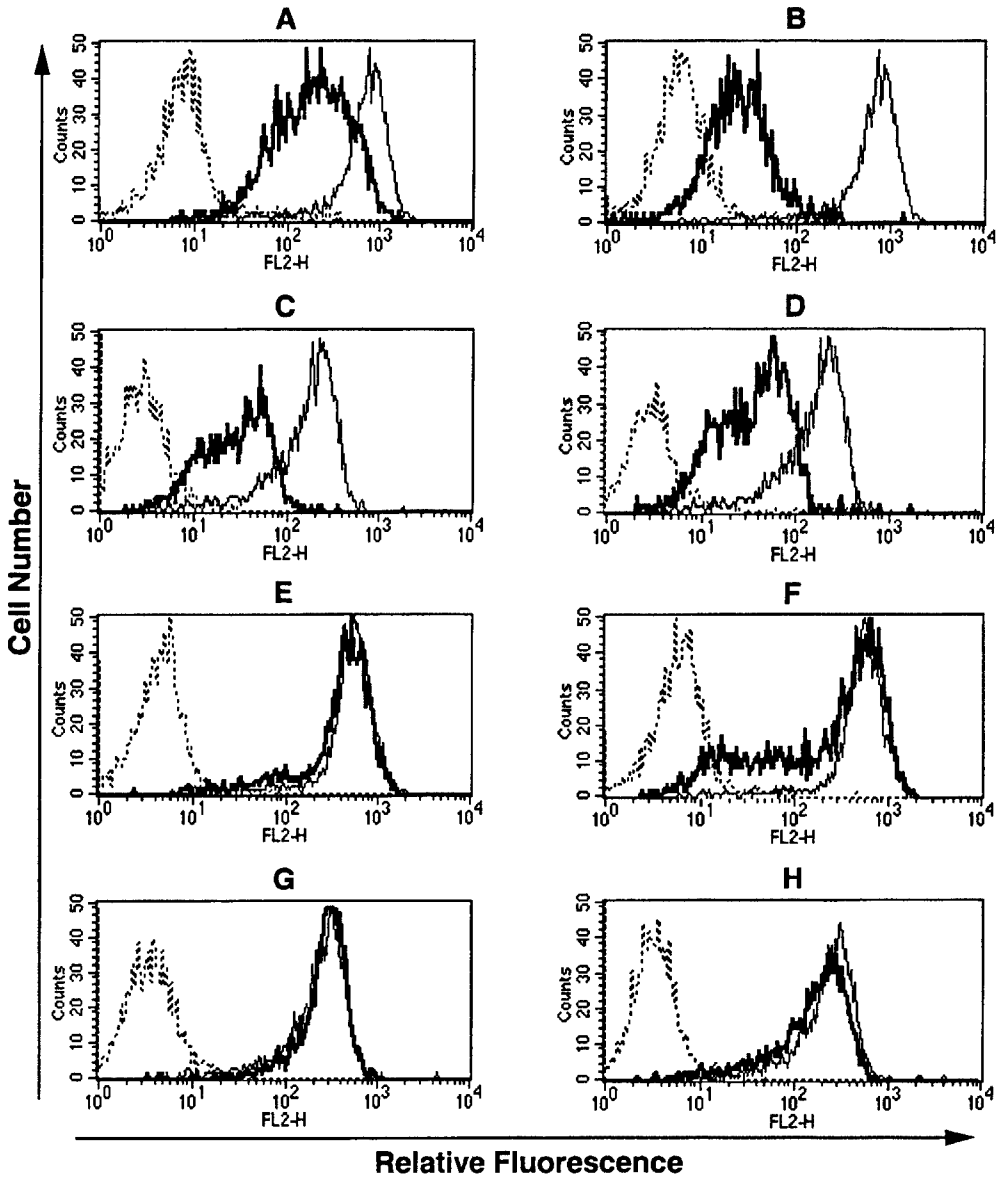

Fig. 7A–H Flow-cytometry analysis of 38C13-HER2/*neu* cells freshly isolated from tumors of 2 mice injected s.c. with 10^4 cells (**A**, **B**) or from cervical metastasis of 2 mice injected i.v. with 10^4 cells (**C**, **D**). All tumors were harvested 15 days after tumor cell injection. The cells were stained with anti-dansyl human IgG3 (- - -) or anti-HER2/*neu* human IgG3 (—), followed by biotinylated goat anti-(human IgG) and phycoerythrin-labeled streptavidin. The expression of human HER2/*neu* was compared with the expression detected in 38C13-HER2/*neu* cells maintained in culture (—). We also tested the expression of HER2/*neu* after the cells had been cultured in vitro for 1 week. **E–H** The cells shown in panels **A–D** respectively after they had been maintained for 1 week in tissue culture

of a relatively small number of cells, but after the tumor becomes established the level of Id protein detected in serum increases at a logarithmic rate making the treatment of established tumors very difficult [28]. The lack of tumor-shed ECD^{HER2} in our HER2/*neu*-expressing cell lines indicates that secretion of this TAA will not interfere with assessment of antibody or antibody fusion protein treatments targeting HER2/*neu*.

In previous studies, we found that expression of human HER2/*neu* on the surface of murine CT26 and MC38 adenocarcinomas and EL4 T-cell lymphoma does not significantly change the in vivo growth properties or morphology of these cells [31]. These in vivo properties dramatically contrast with the cell death and complete tumor regression, resulting in permanent immunity to further challenge observed in s.c. 38C13-HER2/*neu* tumors. This regression was effected through immunological mechanisms since it was not observed in syngeneic immunodeficient mice. These differences in growth characteristics appear to be a consequence of either the nature of the parental cells and/or the mouse strain rather than the level of HER2/*neu* expression, which is similar in all tumor models (data not shown). The same HER2/*neu* expression vector, MoMuLV-based retroviral vector, including the *neo* gene under the control of the SV40 promoter, was used to produce all the HER2/*neu*-expressing cell lines.

The ability to transfer immunity into naïve mice using serum from immune mice suggests that an antibody-mediated mechanism is at least partially responsible for tumor rejection and the maintenance of immunity. This is consistent with other reports that vaccinations using either the Id protein, or DNA encoding for Id, induced tumor protection that can be largely attributed to humoral rather than cellular immunity [9, 40]. We also showed that immunity can be transferred by splenocytes from immune mice; however, this is also consistent with the humoral mechanism because 40% of splenocytes are

B cells [19]. However, as 35% of splenocytes are T cells [19] we can not exclude the possibility that a T cell immune response might also provide antitumor immunity. We have found that transduction of this tumor and other murine tumor models with human HER2/*neu* does not decrease the level of expression of MHC class I (M.L. Penichet et al., unpublished results), suggesting that those cells may be able to elicit targeted cytotoxic T cell response. Further studies are required to define the role (if any) of cellular immunity in tumor rejection and immunity.

Having shown that immunity can be transferred by sera, we characterized the anti-(human HER2/*neu*) and anti-Id responses. We detected an anti-human HER2/*neu* antibody response in mice bearing HER2/*neu*-expressing tumors (s.c. and i.v.). A similar humoral response has also been described in mice bearing primary s.c. or metastatic CT26-HER2/*neu*, MC38-HER2/*neu*, and EL4-HER2/*neu* tumors, although in these models tumor regression was not observed [31]. Although the anti-(human HER2/*neu*) antibodies elicited against the s.c. 38C13-HER2/*neu* tumors may play a role in tumor rejection, we have found no correlation between the level of anti-HER2/*neu* titers and the fate of the tumor (regression or progression). We also found that continuously growing tumors maintain human HER2/*neu* expression, suggesting that variants lacking the expression of human HER2/*neu* are not selected. An alternative possibility is that the presence of foreign antigens such as HER2/*neu* on the surface of 38C13 serves as an adjuvant to facilitate a humoral immune response against other antigens such as the Id. Anti-Id therapy has been successful in the 38C13 model [6, 7, 25, 30]. In fact we have found higher titers of anti-Id antibodies in mice bearing s.c. 38C13-HER2/*neu* than in mice bearing s.c. 38C13 tumors. Furthermore higher anti-Id titers appear to correlate with tumor regression. However anti-Id antibodies were not detected in some of the mice showing regressing s.c. 38C13-HER2/*neu* tumors. It is possible that, in these mice, Id shedding by the 38C13 tumors [28] leads to immune complexes decreasing the concentration of anti-Id antibodies in blood and resulting in underestimation of the anti-Id response.

Although the ability to elicit an anti-Id immune response might explain the spontaneous tumor regression, we can not exclude the possibility that other antibodies with unknown specificity have been elicited and that these are partially or totally responsible for tumor regression and immunity. In favor of this hypothesis is the observation that the rejection of 38C13-HER2/*neu* results in immunity to further challenge not only with the parental cell line 38C13, but also with V1, a cell line that is negative for Id expression [38] and insensitive to treatment with anti-Id antibodies [40]. This observation suggests that antitumor immunity generated after regression of 38C13-HER2/*neu* is directed against one or more common antigens shared by 38C13-HER2/*neu*, 38C13 and V1. Such antigens may be known receptor molecules such as CD19 and CD40, which have been successfully used as targets of antibody-based therapy in B cell lymphoma [43] or may be unknown antigens. If unknown antigens are the targets, hybridomas from splenocytes of immune mice may provide novel and effective antibodies for the therapy of B cell lymphoma. Several mechanisms have been described to explain the antitumor activity of anti-(B cell lymphoma) antibodies such as anti-Id, anti-CD19 or anti-CD40, including antibody-dependent cell-mediated cytotoxicity (ADCC) [6, 7, 25] as well as antibody-mediated inhibition of tumor growth [43]. Further studies are required to define the mechanism of action of the antitumor activity present in the sera of mice that have rejected the 38C13-HER2/*neu* tumor.

Xenogenization is a term used to describe attempts to make tumor cells antigenically foreign to their host [22, 23] and includes the expression of foreign antigens such as viral antigens on the surface of tumor cells to potentiate the host immune reaction against the tumor. The rat fibrosarcoma KMT-17 infected with nonlytic murine leukemia virus (Friend virus), like 38C13-HER2/*neu*, regresses spontaneously in the syngeneic host after an initial period of growth and induces protective immunity to noninfected homologous tumor cells [33]. The mechanism of rejection is not fully understood; however, it was found that surface expression of CE7, a non-viral TAA, is strongly enhanced following viral infection. This enhanced expression may stimulate a strong antitumor response, resulting in acquisition of resistance to parental KMT-17 [33]. Although we did not find that alteration in the expression of the Id in the transductant, we cannot rule out the possibilty that there is enhanced expression of other unknown 38C13 TAA antigens.

In addition to expressing HER2/*neu*, 38C13-HER2/*neu* cells also express the product of the *neo* gene, the neomycin phosphotransferase, and this phosphotransferase activity can induce changes in the cells [44] which might result in higher immunogenicity. In fact, the transduction of 9L rat glioma with a *neo* gene was associated with a decreased in vivo tumor growth in immunocompetent animals, although the mechanism responsible was not defined [41]. Although neomycin phosphotransferase is expressed in the intracellular compartment, it is a bacterial gene product and thus potentially can serve as an immunogen. Even though the *neo* gene has been used in vast numbers of in vivo experiments without eliciting an immune reaction, 1 patient receiving multiple infusions of gene-modified T lymphocytes was shown to develop anti-neo and anti-(herpes thymidine kinase) cell-mediated immune responses that coincided with rapid disappearance of transduced cells in vivo [8]. Human HER2/*neu*, the other xenoantigen that we have expressed, shows more than 90% homology with the rodent HER2/*neu* [46]. Despite this high degree of homology, its expression appears to be sufficient to elicit an anti-(human Ig) humoral immune response and may trigger the antitumor activity. Thus, the antitumor immune reaction may be elicited by increased expression of TAA and/or by the expression of the xenoantigens human HER2/*neu* and/or the *neo* gene

product, indeed, it is possible that the xenoantigens act as adjuvants to potentiate the immune response to other TAAs.

Our data suggest that the parental 38C13 cells can be immunogenic and that a s.c. innoculation of a very high dose of 38C13, such as 10^6 cells, is able to trigger a protective immune response in a subset (10%–20%) of mice. This contrasts with previous observations that s.c. inoculation of increasing numbers of 38C13 tumor cells proportionally shortens the mean survival of injected animals. However, we should stress that these observations were made using doses from 10^3 to 10^4 38C13-tumor cells [6, 7, 25] and we have also found these lower doses to yield tumors in all animals. Apparently a threshold level of antigen must be exceeded in order to elicit a protective immune response. This spontaneous immune reaction against 38C13 appears to be enhanced in the transductants. The xenoantigen(s) role as an adjuvant is supported by the finding of significantly higher titers of anti-Id antibodies in mice bearing s.c. 38C13-HER2/*neu* than in mice bearing s.c. 38C13 tumors.

We speculate that regression of 38C13-HER2/*neu* tumor was observed more frequently with larger tumor cell challenges because increased antigenic stimulation results from larger initial antigenic doses. This strong response may be able to eliminate the tumor before dissemination into the lymph nodes. In contrast, lower doses of cells fail to elicit efficient early stimulation of the immune response, giving the tumors the opportunity to disseminate and colonize most of the lymph nodes leading to their dysfunction. The secondary lymphoid organs are critical for both B- and T-cell-mediated immunity [13] and their dysfunction would decrease the antitumor reaction, allowing the tumor to progress. However, it should also be noted that effective antitumor responses are also not elicited by tumors that do not colonize the lymph nodes. It has been suggested that growing tumors are able to suppress the immune response against them [34]. It is possible that, in the absence of initial strong stimulation, the growing tumor is able to decrease the subsequent immune response to its presence.

Our results suggest that, under certain conditions, expressing antigens of different species (xenoantigens) on B cell lymphomas may be a useful therapeutic approach to eliciting an effective antitumor immune response. This can be achieved by vaccination with tumor cells expressing the xenoantigen, an approach that has shown benefits in ovarian cancer patients [27], or by in vivo transduction of B cell lymphomas with genes encoding xenoantigens. The presence of the foreign antigen on the surface of the B cell lymphomas may induce strong stimulation of the patient's formerly quiescent immune system. Once stimulated, the patient's immune repertoire may be directed against other TAA, resulting in the immune-mediated clearance of all B cell lymphoma cells.

The results obtained with s.c. implantation of cells contrast markedly with those obtained after i.v. injection of 38C13-HER2/*neu*. Following i.v. injection, all mice developed a disseminated malignant disease leading to death. Although the tumors elicited following i.v. injection maintain HER2/*neu* expression, they failed to elicit the strong anti-(human HER2/*neu*) and anti-Id humoral immune response observed in mice bearing s.c. 38C13 HER2/*neu*. This observation may not be surprising because it is well known that the s.c. route is superior to the i.v. route in eliciting an immune response in mice [15]. In addition, the i.v. injection of cells results in metastases to the lymph nodes throughout the body, which may seriously compromise the function of these important secondary immune organs [13] resulting in poor antitumor immunity as discussed above.

38C13-HER2/*neu* may indeed be a useful model for evaluating the immunological efficacy of antibody or antibodies fusion proteins. We have shown that an effective immune response eliminates the tumor. This effective response is usually not elicited in mice injected s.c. with low doses (i.e. 10^3) of tumor cells or in mice injected i.v. with any dose. Tumors elicited following both s.c. and i.v. injection maintain a high level of expression of human HER2/*neu*. In addition, the presence of anti-HER2/*neu* antibodies does not preclude the use of these mice for therapies targeting HER2/*neu* since the treatment can be started shortly after the injection of tumor cells when there is little or no humoral response. Indeed it may be possible to target the tumor in the presence of an anti-HER2/*neu* response since anti-(human carcinoembryonic antigen, CEA) was able to target MC38 transduced with human CEA (MC38-cea2), which elicits a murine anti-(human CEA) response [14]. We also found that both anti-HER2/*neu* scFv and anti-HER2/*neu* IgG3 target CT26-HER2/*neu* growing in immunocompentent mice, a cell line that also elicits a murine anti-(human HER2/*neu*) immune response (M. L. Penichet et al., unpublished results). We thus have the opportunity to use both HER2/*neu* and the Id as targets for our antibody fusion proteins. The challenge now is to develop a strategy that will make it possible to elicit an effective immune response to tumor after i.v. injection and/or to low doses of tumor cells injected s.c.

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Appendix 3

A recombinant IgG3-(IL-2) fusion protein for the treatment of human HER2/*neu* expressing tumors

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Anti-HER2/*neu* therapy of human HER2/*neu* expressing malignancies such as breast cancer has shown only partial success in clinical trials. To expand the clinical potential of this approach, we have genetically engineered an anti-HER2/*neu* human IgG3 fusion protein containing interleukin-2 (IL-2) fused at its carboxyl terminus. Anti-HER2/*neu* IgG3-(IL-2) retained antibody and cytokine related activity. Treatment of immunocompentent mice with this antibody fusion protein resulted in significant retardation in the subcutaneous (s.c.) growth of CT26-HER2/*neu* tumors suggesting that anti-HER2/*neu* IgG3-(IL-2) fusion protein will be useful in the treatment of HER2/*neu* expressing tumors. We also found that fusing IL-2 to human IgG3 results in a significant enhancement of the murine anti-human antibody (MAHA) response.

Keywords: Antibodies, Cytokines, Immunotherapy, Cytotoxicity, Antibody Fusion Protein

1. Introduction

The *HER2/neu* proto-oncogene (also known as *c-erbB-2*) encodes a 185 kDa transmembrane glycoprotein receptor known as HER2/*neu* or p185HER2 that has partial homology with the epidermal growth factor receptor and shares with that receptor intrinsic tyrosine kinase activity [1–3]. It consists of three domains: a cysteine-rich extracellular domain, a transmembrane domain and a short cytoplasmic domain [1–3]. Overexpression of HER2/*neu* is found in 25–30% of human breast cancer and this overexpression is an independent predictor of both relapse-free and overall survival in breast cancer patients [4–7]. Overexpression of HER2/*neu* also has prognostic significance in patients with ovarian [5], gastric [8], endometrial [9], and salivary gland cancers [10]. The increased occurrence of visceral metastasis and micrometastatic bone marrow disease in patients with HER2/*neu* overexpression has suggested a role for HER2/*neu* in metastasis [11,12].

The elevated levels of the HER2/*neu* protein in malignancies and the extracellular accessibility of this molecule make it an excellent tumor-associated antigen (TAA) for tumor specific therapeutic agents. In fact, treatment of patients with advanced breast cancer using the anti-HER2/*neu* antibody, trastuzumab (Herceptin, Genentech, San Francisco, CA) previously known as rhuMAb HER2, directed at the extracellular domain of HER2/*neu* (ECDHER2) [13] can lead to an objective response in some patients with tumors overexpressing the HER2/*neu* oncoprotein [14,15]. However, only a subset of patients shows an objective response (5 of the 43 (11.6%)) [14,15]. Although combination of trastuzumab with chemotherapy enhances its anti-tumor activity (9 of 37 patients with no complete response (24.3%)) [16], improved therapies are still needed for the treatment of HER2/*neu* expressing tumors.

Interleukin-2 (IL-2) is a lymphokine produced by T helper cells which stimulates T cells [17–19] and natural killer (NK) cells [18] and augments antibody dependent cell-mediated cytotoxicity (ADCC) [14,15]. Although it was possible to stimulate an anti-tumor response using high doses of systemically administered

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recombinant human IL-2 (rhuIL-2) [21], the systemic administration of high-dose IL-2 had severe toxic side effects [21,22]. Targeting IL-2 to the site of a tumor with an antibody recognizing a tumor associated antigen is one means of achieving locally high concentrations of IL-2 without toxicity [23–26].

We have now expanded the family of antibody-(IL-2) fusion proteins by developing an anti-HER2/*neu* IgG3-(IL-2) fusion protein protein that may provide an effective alternative for the therapy of HER2/*neu* expressing tumors. This novel antibody fusion protein is composed by a human IgG3 with the variable region of the humanized anti-HER2/*neu* antibody, trastuzumab (Herceptin, Genentech, San Francisco, CA) [13–15] genetically fused to human IL-2. In this report we describe and discuss the strategy of construction and the properties of this novel antibody-(IL-2) fusion protein.

2. Materials and methods

Cell lines: CT26-HER2/*neu* was developed in our laboratory by transduction of CT26 cells with the cDNA encoding human HER2/*neu* [14,15]. It was cultured in Iscove's Modified Dulbecco's Medium IMDM (GIBCO, Grand Island, NY) supplemented with 5% bovine calf serum (HyClone, Logan, UT). CTLL-2, an IL-2 dependent murine T cell line (provided by Dr. William Clark, UCLA, CA) was cultured in RPMI 1640 (GIBCO) supplemented with 10% bovine calf serum and IL-2.

Mice: Female BALB/c mice 6–8 weeks of age were obtained from Taconic Farms, Inc. (Germantown, NY).

Vector construction, transfection and initial characterization of anti-human HER2/neu IgG3-(IL-2): For the construction of the heavy chain of anti-human HER2/*neu* IgG3-(IL-2), the DNA encoding the variable region of trastuzumab [13,15] was joined to a human $\gamma 3$ heavy chain containing IL-2 fused at the carboxy terminus of the C_H3 domain. It was expressed with the corresponding anti-HER2/*neu* kappa light chain in P3X63Ag8.653. Stable transfectants were selected and characterized as previously described [13,15]. The fusion protein was purified from culture supernatants using protein A immobilized on Sepharose 4B fast flow (Sigma Chemical, St. Louis, MO). Purity and integrity were assessed by Coomassie blue staining of proteins separated by SDS-PAGE. The ability of the fusion protein to recognize antigen was assessed by flow cytometry using CT26-HER2/*neu* cells. The ability of the fusion protein to support the growth of the IL-2 dependent cell line CTLL-2 was determined as previously described [29].

Immunotherapy: 10^6 CT26-HER2/*neu* cells in 0.15 ml HBSS were injected subcutaneously (s.c.) into the right flank of syngeneic BALB/c mice. Beginning the next day mice randomized into groups of 8 received five daily intravenous (i.v.) injections of 0.25 ml of PBS containing 20 μg of anti-HER2/*neu* IgG3-(IL-2), the equivalent molar amount of anti-HER2/*neu* IgG3, or nothing. Tumor growth was monitored and measured with a caliper every three days until day 15 at which time mice were euthanized. Blood samples were collected, serum was separated from clotted blood and stored at $-20°C$ until assayed by ELISA.

Determination of murine anti-human HER2/neu antibodies: Sera from each treatment group were analyzed by ELISA for the presence of antibodies to human IgG3 using 96-well microtiter plates coated with 50 μl of anti-human HER2/*neu* IgG3 at a concentration of 1 μg/ml. Alkaline phosphatase (AP)-labeled goat anti-mouse IgG (Sigma Chemical, St. Louis, MO) or rat antibodies specific for murine IgG2a, IgG2b, IgG3, IgG1 or kappa (Pharmingen, San Diego, CA) followed by alkaline phosphatase (AP)-labeled goat anti-rat IgG (Pharmingen, San Diego, CA) were used to detect bound murine antibodies. All ELISAs for comparison of titers between the experimental groups were made simultaneously in duplicate using an internal positive control curve for each plate.

Statistical analysis: Statistical analysis of the titration ELISA and anti-tumor experiment was done using a two-tailed Student's t-test. For all cases results were regarded significant if p values were $\leqslant 0.05$.

3. Results

Anti-HER2/*neu* IgG3-C_H3-(IL-2) was constructed and expressed as previously described for similar IL-2 fusion proteins [23]. Heavy and light chains of the expected size were synthesized, assembled and secreted. The fusion protein specifically bound to the human HER2/*neu* expressed on the surface of the murine cell line CT26-HER2/*neu* and was able to stimulate the proliferation of the IL-2 dependent cell line CTLL-2 with a similar proliferative response observed with equimolar IL-2 concentrations of rhuIL-2 and anti-HER2/*neu* IgG3-(IL-2) (data not shown).

To investigate in vivo anti-tumor activity, 10^6 CT26-HER2/*neu* cells were injected s.c. into the right flank of BALB/c mice. Beginning the next day mice were

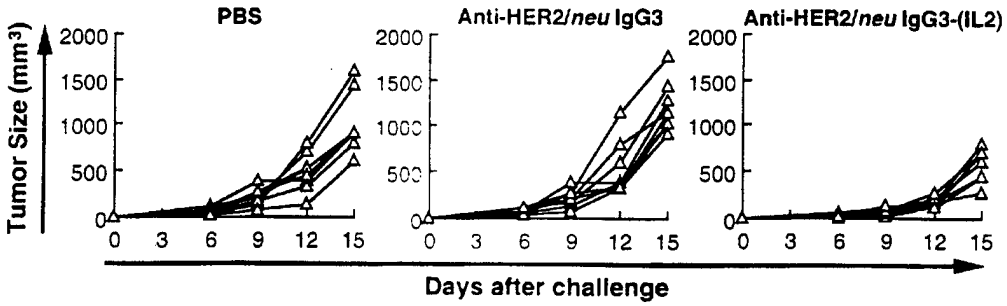

Fig. 1. Anti-Tumor Activity of anti-HER2/*neu* IgG3-(IL-2) and anti-HER2/*neu* IgG3. 10^6 CT26-HER2/*neu* cells were injected s.c. into the right flank of BALB/c mice. Beginning the next day groups of 8 mice received five daily i.v. injections of 0.25 ml of PBS containing 20 μg of anti-HER2/*neu* IgG3-(IL-2), the equivalent molar amount of anti-HER2/*neu* IgG3 or nothing. Tumor growth was measured with a caliper every three days until day 15. The volume was calculated for each mouse of each treatment group, PBS (panel A), anti-HER2/*neu* IgG3 (panel B), and anti-HER2/*neu* IgG3-(IL-2) (panel C).

randomized and groups of 8 received five daily i.v. injections of 0.25 ml of PBS containing 20 μg of anti-HER2/*neu* IgG3-(IL-2), the equivalent molar amount of anti-HER2/*neu* IgG3 or nothing. Tumor growth was monitored and measured with a caliper every three days until day 15 at which time mice were euthanized and serum samples collected. Injection of anti-HER2/*neu* IgG3- (IL-2) results in a significant retardation in the tumor growth in most of the mice as compared with the respective controls of PBS or anti-HER2/*neu* IgG3 (Fig. 1). A two-tailed Student's t-test comparing the tumor volume for each mouse from the group treated with anti-HER2/*neu* IgG3-(IL-2) fusion protein with the mice from the group treated with PBS or anti-HER2/*neu* IgG3 showed that the tumor sizes were statistically different ($p < 0.05$) for all the observed points: days 6, 9, 12, and 15 (Table 1). There was no statistically significant difference in tumor volume between the groups injected with PBS and anti-HER2/*neu* IgG3.

Mice treated with anti-HER2/*neu* IgG3-(IL-2) exhibited a significantly increased ($p < 0.01$) antibody response to anti-HER2/*neu* human IgG3 compared to mice treated with anti-HER2/*neu* IgG3 (Table 2). Mice treated with anti-HER2/*neu* IgG3-(IL-2) showed higher levels of antibodies of all isotypes recognizing human IgG3 when compared to anti-HER2/*neu* IgG3 treated mice (Fig. 2).

4. Discussion

In an attempt to improve the clinical efficacy of anti-HER2/*neu* based therapies we have developed an alternative approach in which human IgG3 containing the variable regions of trastuzumab has been genetically fused to immunostimulatory molecules such as the cytokine IL-12 [30], the costimulatory molecule B7.1 [31], and now IL-2. Targeting IL-2 to the site of a tumor with an antibody-(IL-2) fusion proteins recognizing TAAs has been an effective approach to specifically eliminate many tumors [23,26].

A number of factors were considered in the design of our anti-HER2/*neu* IgG3-(IL-2) fusion protein. Human IgG3 was chosen because its extended hinge region should provide spacing and flexibility thereby facilitating simultaneous antigen and receptor binding [32,33] IgG3 is also effective in complement activation [34], and binds FcγRs [34]. IL-2 was used because of its potent immunostimulating properties [17–20,35] and because targeting IL-2 to the site of a tumor with an antibody-(IL-2) fusion protein recognizing TAAs has been an effective approach for specifically eliminating many tumors [23–26]. Antibody-(IL-2) fusion proteins recognizing TAAs have shown superior anti-cancer activity compared with an equivalent amount of free antibody and IL-2 or non-tumor specific antibody cytokine fusion proteins [23,26]. Human IL-2 was used so that the resulting fusion protein was mostly human. Human IL-2 is active in mice making it possible to perform in vivo studies using immune competent mice bearing human HER2/*neu* expressing tumors.

A a single chain Fv specific for peptide epitopes of HER2/*neu* presented by HLA-A*0201 molecules genetically fused to IL-2 [36] (neu-Ab-IL-2) was found to enhance tumor cell eradication by HER2/*neu*-specific $CD8^+$ T cells in an adoptive transfer model in SCID mice. Surprisingly, the combination of non-tumor-specific $CD8^+$ T cells and fusion protein also induced a significant delay of tumor growth, suggesting the potential use of this molecule for redirecting non-tumor-specific T cells to eliminate tumors [36]. However,

Table 1
Mean tumor volumes and statistical significance

Days after the Challenge	Mean Tumor Volumes (mm^3)[a]			Significance[b]	
	PBS	IgG3	IgG3-IL-2	(p) 1	(p) 2
6	67	83	23	0.0114	0.0001
9	221	221	80	0.0070	0.0007
12	479	535	188	0.0013	0.0063
15	1006	1217	571	0.0054	0.0001

[a] 10^6 CT26-HER2/*neu* cells were injected s.c. into the right flank of BALB/c mice. Beginning the next day groups of 8 mice received five daily i.v. injections of 0.25 ml of PBS containing 20 μg of anti-HER2/*neu* IgG3-(IL-2), the equivalent molar amount of anti-HER2/*neu* IgG3 or nothing. Tumor growth was measured with a caliper every three days until day 15 and the volume was calculated for each mouse of each treatment group. Mean Tumor Volumes respresents the average tumor volume for each treatment group.
[b] Statistical analysis of the anti-tumor experiments was done using a two-tailed Student's t-test. For all cases results were regarded significant if p values were $\leqslant 0.05$. (p) 1 and (p) 2 represent the p obtained when Mean Tumor Volumes of the group injected with anti-HER2/*neu* IgG3-(IL-2) were compared with PBS and anti-HER2/*neu* IgG3 controls respectively.

Table 2
Murine anti-human IgG3 titers[a]

Treatment	Mouse Number							
	1	2	3	4	5	6	7	8
IgG3	450	450	150	150	450	150	150	150
IgG3-(IL2)	1350	1350	450	1350	450	450	450	450

[a] Groups of 8 mice injected s.c. with 10^6 CT26-HER2/*neu* cells were treated beginning the next day with five daily i.v. injections of 0.25 ml of PBS containing 20 μg of anti-HER2/*neu* IgG3-(IL2), the equivalent molar amount of anti-HER2/*neu* IgG3 or nothing. Mice were bled 15 days after the injection of the tumor cells and the sera analyzed by a titration ELISA using plates coated with human IgG3. The presence of antibodies was detected using AP-labeled anti-mouse IgG. Values represent the average of duplicate dilutions of serum required to yield an absorbance of 0.1 (410 nm) after 1 hr of incubation.

in contrast with immunoglobulins such as IgG3, scFvs do not have an Fc. Fc associated funtions such as ADCC (an activity that can be enhanced by the presence of IL-2) may play a role in the anti-tumor activity of recombinant antibodies or antibody-(IL-2) fusion proteins. In fact, ADCC has been proposed as a possible mechanism for the clinical response observed with trastuzumab (Herceptin, Genentech, San Francisco, CA) [15]. In addition, studies from other laboratories have shown that while a mouse-human chimeric anti-Id IgG1-mouse IL2 fusion protein (chS5A8-IL2) was effective in the in vivo eradication of the 38C13 tumor, an anti-Id scFv-IL2 fusion protein (scFvS5A8-IL2) containing the variable regions of the chS5A8-IL2 failed to confer protection. These studies suggested that the Fc effector functions such as ADCC contributed to the anti-tumor activity against 38C13 [24]. It is therefore possible that an anti-HER2/*neu* IgG3-(IL-2) will be superior to anti-HER2/*neu* scFv-(IL-2) in its anti-tumor activity.

We have found that treatment with anti-HER2/*neu* IgG3-(IL-2) causes a significant retardation in the growth of s.c. CT26-HER2/*neu* tumors under conditions in which anti-HER2/*neu* failed to confer protection. However, we did not observe complete tumor eradication in any mice. Several factors could explain the failure to obtain complete tumor remission. The dose, route and schedule of treatment (daily i.v. injection of 20 μg for 5 days) may not be the optimal and/or the tumor model may not be ideal. In addition, we found that treatment with anti-HER2/*neu* IgG3-(IL-2) results in a dramatic increase in the murine anti-human antibody (MAHA) response. This humoral immune response may be sufficient to neutralize multiple injections of the antibody fusion protein.

It is possible that that an anti-human IgG immune response will not pose a problem in humans, and anti-HER2/*neu* IgG3-(IL-2) may be even more effective in patients than in mice. However, the IL-2 in the antibody fusion protein may act as an adjuvant to elicit an im-

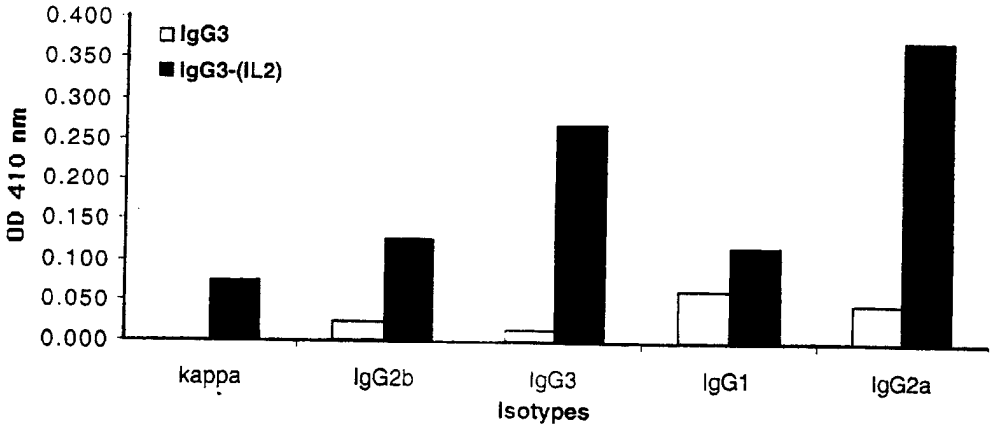

Fig. 2. Isotype profile of antibodies specific for human IgG3. Pooled sera (1/50 dilution) from mice treated with anti-HER2/*neu* IgG3 (clear bar), or anti-HER2/*neu* IgG3-(IL-2) (black bar) were analyzed by ELISA for antibodies of different isotypes recognizing anti-HER2/*neu* IgG3.

mune response against the variable regions of humanized or chimeric antibodies. In addition, even though each component of the antibody-(IL-2) fusion protein may not be antigenic by itself in humans, the novel combination of components may produce neoantigenic determinants that will elicit an immune response. Although in certain cases this enhancement of the immune response may be a serious drawback for the therapeutic use of antibody-(IL-2) fusion proteins, in other cases it may be irrelevant [37] or even an advantage [38,39].

In conclusion, our results suggest that an anti-HER2/*neu* IgG3-(IL-2) fusion protein containing human IL-2 may be an effective therapeutic in patients with tumors overexpressing HER2/*neu*. The combination of an anti-HER2/*neu* antibody with IL-2 yields a protein with the potential to eradicate tumor cells by a number of mechanisms including the down regulation of HER2/*neu* expression, ADCC and the stimulation of a strong anti-tumor immune response through the immunostimulatory activity of IL-2. In addition, the anti-HER2/*neu* IgG3-(IL-2) fusion protein may be effective against tumor cells which express a truncated form of ECD^{HER2} lacking the receptor function rendering them particularly resistant to anti-HER2/*neu* antibody therapy [14]. Because of IL-2's ability to elicit an immune response to associated antigens (as observed with the dramatically increased immune response against human IgG3), it is also possible that association of anti-HER2/*neu* IgG3-(IL-2) with soluble ECD^{HER2} shed by tumor cells will enhance the anti-tumor immune response against ECD^{HER2}. Secretion of ECD^{HER2} has been reported to be a serious drawback for anti-HER2/*neu* therapy in humans [14,15].

Finally we would like to stress that anti-HER2/*neu* IgG3-(IL-2) would not be a replacement for trastuzumab (Herceptin, Genentech, San Francisco, CA), but instead would provide an alternative therapy to be used in combination with the antibody or other anti-cancer approaches. These approaches might include chemotherapy or other anti-HER2/*neu* antibody fusion proteins such as anti-HER2/*neu* with the costimulator B7.1 [31] or the cytokine IL-12 [30]. The availability of more than one antibody fusion protein will allow us to explore potentials synergistic effects that may be obtained from manipulating the immune response.

Acknowledgments

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Appendix 4

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Mary Ann Liebert, Inc.

Mechanism of Antitumor Activity of a Single-Chain Interleukin-12 IgG3 Antibody Fusion Protein (mscIL-12.her2.IgG3)

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ABSTRACT

We have constructed an antibody interleukin-12 (IL-12) fusion protein (mscIL-12.her2.IgG3) that demonstrates significant antitumor activity against the murine carcinoma CT26-expressing human HER2/*neu*. We now report that this antitumor activity is dose dependent and comparable to or better than recombinant murine IL-12 (rMuIL-12) using subcutaneous and metastatic models of disease. The antitumor activity of mscIL-12.her2.IgG3 is reduced in Rag2 knockout mice, suggesting that T cells play a role in tumor rejection. In SCID-beige mice, the antitumor activity is further reduced, suggesting that natural killer (NK) cells or macrophages or both are also important. The isotype of the antibody response to HER2/*neu* is consistent with a switch from a Th2 to a Th1 immune response and the infiltration of mononuclear cell in tumors from mice treated with mscIL-12.her2.IgG3. Immunohistochemistry reveals that mscIL-12.her2.IgG3 is antiangiogenic. Thus, the mechanism of the antitumor activity exhibited by mscIL-12.her2.IgG3 is highly complex and involves a combination of T and NK cell activity, a switch to a Th1 immune response, and antiantiogenic activity. This is the first study comparing the *in vivo* antitumor activity of an antibody-IL-12 fusion protein and free IL-12. Our results suggest that antibody-IL-12 fusion proteins may be useful for the treatment of human cancer.

INTRODUCTION

INTERLEUKIN-12 (IL-12), a heterodimeric cytokine released by professional antigen-presenting cells (APC), promotes cell-mediated immunity by inducing naive $CD4^+$ T cells to differentiate into Th1 cells.[1–3] In addition, IL-12 has the ability to enhance the cytotoxicity of natural killer (NK) and $CD8^+$ T cells.[2,4] Moreover, the interferon-γ (IFN-γ) produced by IL-12-stimulated T and NK cells can retard tumor growth by eliciting an inhibition of tumor angiogenesis[2,5,6] and enhancing immune recognition of tumor cells through upregulated MHC expression.[7] IL-12 does not have a direct antiproliferative effect on tumor cells *in vitro*, although it may inhibit tumor cell attachment to matrices and growth factor-induced invasion.[8]

Systemic administration of IL-12 to mice bearing subcutaneous (s.c.) tumors results in dose-dependent tumor growth inhibition, prolongation of survival, and even tumor regression in some models.[8–10] Treatment with IL-12 has been demonstrated to inhibit established experimental pulmonary and hepatic metastases and to reduce spontaneous metastases.[8–11] Clinical trials using IL-12 in patients with cutaneous T cell lymphoma, renal cell carcinoma, and melanoma have demonstrated efficacy.[12–15] However, the systemic administration of recombinant IL-12 (rIL-12) in humans has been limited by severe toxicity, making it impossible to achieve an effective dose at the site of the tumor.[2,13,16] Ideally, strategies that increase the cytokine concentration at the site of the tumor and allow for lower systemic levels should be more effective.

Antibody-IL-12 fusion proteins in which tumor-specific antibodies can be used to selectively target IL-12 to tumors provide an attractive delivery vehicle. The specific targeting should make it possible to achieve effective doses at the site of the tumor without accompanying systemic toxicity. However, IL-12 is a disulfide-linked heterodimer of two subunits p35 and p40 and requires the expression of two separate genes and correct heterodimer assembly for activity.[17] To address this issue, Gillies et al.[18] constructed an antibody-IL-12 fusion protein specific for the pancarcinoma antigen EpCAM, in which the

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p35 subunit was fused to the carboxy-terminus of a human IgG1 heavy chain. The p40 subunit was expressed as a separate polypeptide, which must then assemble with the p35 subunit. A construct composed of murine p35 and human p40 proved to be highly effective at treating severe combined immunodeficient (SCID) mice with established pulmonary metastasis of the CT26 carcinoma-expressing human EpCAM[18] despite the fact that this antibody-IL-12 fusion protein was less active than murine IL-12 (MuIL-12).

As an alternative approach, we have constructed an antibody-IL-12 fusion protein, mscIL-12.her2.IgG3, in which single-chain MuIL-12 (mscIL-12) was joined to the amino-terminus of an IgG3 antibody by a flexible Gly-Ser linker.[19] The IL-12 in this fusion protein has been modified in that the two IL-12 subunits are covalently linked and this scIL-12 is further tethered to a large antibody molecule, making it of interest to determine the mechanisms of the antitumor activity of the IL-12 in the fusion protein.[19] The mscIL-12.her2.IgG3 contains the variable region of trastuzumab (Herceptin) (Genentech, San Francisco, CA) specific for the extracellular domain of HER2/*neu* (ECD^{HER2}),[20] a tumor-associated antigen (TAA) the overexpression of which is associated with poor prognosis in many different cancers, including breast, ovarian, lung, and gastric.[21–24] Although treatment of HER2/*neu*-expressing tumor with trastuzumab has shown some efficacy,[25,26] improved therapies are still needed for the treatment of HER2/*neu*-expressing tumors.

Although our long-term goal was to improve the antitumor effect of trastuzumab by the production of an antibody-IL-12 fusion protein for therapeutic use in humans, MuIL-12 was used for these initial studies because it has activity on both human and murine cells, whereas human IL-12 (HuIL-12) has activity only on human cells.[19] The use of MuIL-12 makes it possible to both test biologic activity using human peripheral blood mononuclear cells (PBMC) and perform *in vivo* studies to examine the effects against HuHER2/*neu*-expressing murine tumors using immunocompetent mice. We found that mscIL-12.her2.IgG3 retains antigen specificity and IL-12 bioactivity *in vitro* and demonstrates antitumor activity in BALB/c bearing s.c. CT26-HER2/*neu* tumors under conditions in which treatment with anti-HER2/*neu* IgG3 failed to confer protection. Because the ultimate goal is to use mscIL-12.her2.IgG3 as an antitumor agent and the mechanism of IL-12 antitumor activity appears to depend on many variables, including the dosing regimen, it was important to determine the mechanism of the antitumor activity of the antibody fusion proteins and compare it with that of IL-12. It should be noted that in our previous studies[19] and in the report of Gillies et al.[18] a control of free IL-12 was not included in the animal experiments, making it impossible to determine if the antibody-IL-12 fusion protein differs in activity from free IL-12.

In the present studies, we expanded the characterization of mscIL-12.her2.IgG3 by studying its antitumor activity in immunocompetent BALB/c mice using CT26-HER2/*neu* models previously developed in our laboratory.[27] In addition, we directly compared the activity of mscIL-12.her2.IgG3 with that of equivalent doses of free recombinant MuIL-12 (rMuIL-12). We show that antitumor activity of mscIL-12.her2.IgG3 is highly complex and reflects, among other things, a switch to a Th1 response, tumor infiltration by T and NK cells, and antiangiogenic activity.

MATERIALS AND METHODS

Cell lines and reagent

CT26, a murine colon carcinoma induced in BALB/c mice by intrarectal injection of N-nitroso-N-methylurethane,[28] was kindly provided by Dr. Young Chul Sung (Pohang University of Science and Technology, Korea). CT26-HER2/*neu* cells were developed in our laboratory by transduction of CT26 cells with the cDNA for HER2/*neu*.[27] Both cell lines were cultured in IMDM (Irvine Scientific Inc., Irvine, CA) supplemented with 5% bovine calf serum (Atlanta Biologicals, Norcross GA) at 37°C with 5% CO_2. mscIL-12.her2.IgG3 was purified from culture supernatants as previously described.[19] rMuIL-12 was kindly provided by Dr. Stanley Wolf (Genetics Institute, Cambridge, MA).

Mice

Female 6–8-week-old BALB/c and SCID-beige (C.B-17 SCID-beige) mice were obtained from Taconic Farms, Inc. (Germantown, NY). Female 6–8-week-old $Rag2^{-/-}$ knockout mice (129 Rag2) were kindly provided by Colin McLean at UCLA. All mice were conventionally housed, and all animal experiments were approved by the UCLA Review Board of Animal Research Committee.

Experimental metastatic model and antitumor treatment

On day 0 of the experiment, female 6–8-week-old BALB/c mice (8 mice per treatment arm) were injected i.v. with a single cell suspension of 5×10^4 CT26-HER2/*neu* cells resuspended in 0.3 ml Hank's balanced salt solution (HBSS) (GIBCO-BRL, Grand Island, NY) to induce pulmonary metastases. Mice were mixed, randomly segregated into the indicated groups, and on days 3–7 of the experiment, treated with daily i.v. injections of phosphate-buffered saline (PBS) or PBS containing 1 μg IL-12 equivalent of mscIL-12.her2.IgG3 or 1 μg rMuIL-12. On day 16, each mouse was killed, the lungs were removed, and the number of metastases was determined using the procedure of Wexler,[29] with modifications. The trachea was exposed, a small incision was made, and India ink (15%) was slowly pushed into the lungs through a needle and syringe inserted into the trachea. The lungs were removed and placed in water. The lungs were blotted on a paper towel and placed in Bouin's fixative solution (Ricca Chemical Co., Arlington, TX) overnight. The lungs were washed three times with 70% ethanol. The number of metastases was counted under a dissecting microscope. A control of anti-HER2/*neu* IgG3 was not included in this experiment because we have previously observed that lung metastases of CT26-HER2/*neu* cells are resistant to the treatment with antibody alone (anti-HER2/*neu* IgG3) at a dose greater than that used in the present study (L.S. Peng et al., unpublished observations).

Subcutaneous tumor model and antitumor treatment

Female 6–8-week-old BALB/c, Rag2 knockout, or SCID-beige (6–8 mice per treatment arm) were injected s.c. in the right flank with a single cell suspension of 1×10^6 CT26 or

CT26-HER2/*neu* cells resuspended in 0.15 ml HBSS on day 0 of the experiment. The mice were mixed, separated randomly into the indicated groups, and on days 6–10 of the experiment treated with daily i.v. injections of PBS or PBS containing 1 or 5 μg IL-12 equivalent of mscIL-12.her2.IgG3 or 1 or 5 μg rMuIL-12. The tumor size was monitored by caliper measurement every other day, and tumor volume was calculated. On days 20–22, mice were killed, and tumor and sera were harvested for subsequent studies. Tumors were processed for histologic and immunohistochemical analysis. Blood samples were collected, and serum was separated from clotted blood and stored at −20°C until assayed by ELISA. The control of anti-HER2/*neu* IgG3 was not included in these experiments because we have previously observed that s.c. CT26-HER2/*neu* tumors are resistant to treatment with anti-HER2/*neu* IgG3.[19]

Tumor histology

Immediately after harvest, tumors were frozen in OCT embedding compound (Miles Inc., Elkhart, IN). Tumor sections were cut to 6 μm, fixed in 10% formalin, washed, then immersed in hematoxylin aqueous formula (Biomeda, Foster City, CA) for 5 min. The slides were washed and immersed in Scott's water (Fisher Chemical, Somerville, NJ) for 1 min and then washed and immersed in Eosin Y solution alcoholic with phloxine (Sigma Chemical, St. Louis, MO) for 1 min, 15 sec. The slides were dehydrated in ethanol, cleared in xylene, and mounted with Permount (Fisher Chemical).

*Time course of early response in mice bearing s.c. CT26-HER2/*neu *tumors*

On day 0 of the experiment, 6–8-week-old female BALB/c mice were injected with 1×10^6 CT26-HER2/*neu* cells per mouse s.c. in the right flank. Twenty-six mice were used in the experiment, with 2 mice in the pretreatment and PBS-treated groups and 3 mice in all other treatment groups. The mice were treated with PBS or 1 μg IL-12 equivalents per day of mscIL-12.her2.IgG3 or rMuIL-12 for 5 days beginning on day 6 after tumor inoculation. The mice were not treated if they were to be killed that day. The mice were killed at four different time points: 24 h before treatment was initiated (pretreatment group) and 24 h, 4 days, and 7 days after treatment was initiated. At each time point, blood was collected, and the tumor and liver were harvested.

Anti-CD3/anti-NK1.1 cell staining

To determine the phenotype of the lymphocytes present in tumor-bearing animals, $0.5–1 \times 10^6$ lymphocytes isolated from the livers were added to 2 ml PBS mix (PBS, 0.1% NaN_3, 2% bovine calf serum). The tubes were centrifuged for 5 min at 180*g* at 4°C, decanted, and drained on 3-mm Whatman filter paper (Clifton, NJ). The cells were incubated with 100 μl hamster antimouse CD3 and biotinylated antimouse NK1.1 for 2 h at 4°C with agitation. All antibodies were used at a concentration of 1 μg/100 μl in PBS mix and were purchased from PharMingen (San Diego, CA). A hamster IgG isotype-matched nonspecific antibody and PBS mix were used as controls for the anti-CD3 and the anti-NK1.1 antibodies, respectively. After 2 ml PBS mix was added, each sample was centrifuged for 5 min at 180*g* at 4°C, decanted, drained on filter paper, and washed with another 2 ml PBS mix. The samples were then incubated in diluted FITC-conjugated antihamster antibody and diluted streptavidin-phycoerythrin (PE) at 0.5 μg/1500 μl (PharMingen) for 45 min at 4°C with agitation. PBS mix (2 ml) was added to each sample. Samples were centrifuged for 5 min at 180*g* at 4°C, decanted, and drained on filter paper. Paraformaldehyde (200 μl 2%) was added to each sample to fix the cells, and the samples were stored covered at 4°C until they could be analyzed by flow cytometry. Analysis was performed with a FACScan (Becton Dickinson, Mountain View, CA) equipped with a blue laser excitation of 15 mW at 488 nm. The two fluorochromes, FITC and PE, were electronically compensated using singly labeled spleen cells.

*Anti-HER2/*neu *ELISA*

Immulon 2 96-well plates (Dynex Technologies Inc., Chantilly, VA) were coated (50 μl/well) with ECD^{HER2} (1 μg/ml) in carbonate buffer, pH 9.6, and incubated at 4°C overnight. The plates were washed five times with PBS and blocked overnight at 4°C with 100 μl/well 3% bovine serum albumin (BSA) in PBS + 0.02% NaN_3. The plates were washed five times with PBS. Serum (pooled from a treatment group, diluted 1:50 in 1% BSA in PBS) was added at 50 μl/well in triplicate. The plates were incubated overnight at 4°C, then washed five times with PBS. Rat antibodies specific for MuIgG1 and MuIgG2a (PharMingen) were diluted 1:5 in 1% BSA in PBS and added at 50 μl/well to the plates. The plates were incubated at ambient temperature for 2 h and washed five times with PBS. Goat-anti-rat antibody diluted 1:1000 in 1% BSA in PBS conjugated to alkaline phosphatase (PharMingen) was added at 50 μl/well, and the plates were incubated at 37°C for 1 h. The plates were washed five times with PBS, 50 μl/well phosphatase substrate (1 tablet *p*-nitrophenyl phosphate [Sigma] per 5 ml diethanolamine buffer [49 ml diethanolamine + 120 μl 1 M $MgCl_2$ + dH_2O to 500 ml, pH 9.8]) were added, and the plates were read at 410 nm after developing 1–2.5 h at ambient temperature.

Antiangiogenic activity

Immediately after harvest, tumors were frozen in OCT embedding compound. Tumor sections were cut to 6 μm and fixed in acetone. The slides were rehydrated in PBS, pH 7.5 (three changes in 5 min). Any endogenous peroxidase activity was quenched by immersing the slides in 0.3% H_2O_2 in methanol for 30 min. The slides were washed in PBS, pH 7.5 (three changes in 5 min). The sections were blocked in blocking buffer (3% BSA in PBS, pH 7.4) for 20 min in a humidified chamber. The blocking buffer was aspirated off the slides, and rat antibodies to murine PE-CAM (PharMingen), an adhesion molecule (CD31) on endothelial cells, diluted to 0.5 μg/ml in blocking buffer, were added to the sections. After overnight incubation in a humidified chamber at ambient temperature, the slides were washed in PBS, pH 7.5 (three changes in 5 min). Diluted biotinylated antirat antibody, mouse adsorbed (Vector Labs, Burlingame, CA), was added to the sections at 2.5 μg/ml. After 1 h incubation in a humidified chamber at ambient temperature, the slides were washed in PBS, pH 7.5 (three changes in 5 min). Vectastain Elite ABC (Vector Labs) (2 drops A and 2 drops B per 5 ml PBS, pH 7.5, mixed 30 min before use) was added to the slides, and they were placed in a humidified cham-

ber for 30 min at ambient temperature. The slides were washed in PBS (three changes in 5 min), SG substrate (Vector Labs) (3 drops chromagen and 3 drops H_2O_2 solution per 5 ml PBS, pH 7.5) was added, and this allowed to develop for 10 min. The slides were washed in PBS, pH 7.5 (three changes in 5 min), followed by a short dip in dH_2O. Nuclear fast red counterstain (Vector Labs) was added to the sections and allowed to develop for 10 min. After washing in three changes of tap water, the slides were dehydrated in successive incubations in 95% ethanol (three times, 2 min each) and 100% ethanol (three times, 2 min each). The slides were then cleared in seven changes of xylene and mounted with Permount (Fisher Chemical).

Photographs of the sections (at least three fields per tumor section, average five) were taken, the photographs were overlaid with a 5×5-μm grid, and vessel intersections were counted. The number of intersections per view was averaged for all tumors within the same treatment group at the same time point, and standard deviations (SD) were calculated.

Statistical analysis

Statistical analysis of the antitumor experiments was done using a two-tailed Student's *t*-test. Results were regarded significant if p values were ≤ 0.05.

RESULTS

*Antitumor activity of rMuIL-12 and mscIL-12.her2.IgG3 in BALB/c mice bearing CT26-HER2/*neu *pulmonary metastases*

Because one of the potential applications of an antibody fusion protein would be to target metastatic disease, we examined the antitumor activity of mscIL-12.her2.IgG3 in an experimental CT26-HER2/*neu* lung metastasis model. Figure 1 shows that treatment of mice with rMuIL-12 or the equivalent IL-12 amount of mscIL-12.her2.IgG3 caused a potent inhibition in the number of CT26-HER2/*neu* pulmonary metastases (mean number of metastases 20 and 22, respectively) compared with PBS-treated controls (mean 88). In both cases, this difference was statistically significant, $p = 0.019$ for rMuIL-12 and $p = 0.025$ for mscIL-12.her2.IgG3. Although there was no significant difference between rMuIL-12 and mscIL-12.her2.IgG3, we observed that whereas the response of mice to treatment with rMuIL-12 was rather homogeneous, animals treated with mscIL-12.her2.IgG3 were split in two subgroups, one subgroup showing fewer metastases than the best responders of the group treated with rMuIL-12, and the other subgroup showing more pulmonary metastases than the mice treated with rMuIL-12.

*Dose-dependent antitumor activity of rMuIL-12 and mscIL-12.her2.IgG3 in BALB/c mice bearing s.c. CT26-HER2/*neu *tumors*

In our previous experiments using mscIL-12.her2.IgG3 at 1 μg/day for 5 days, we observed tumor growth arrest when the fusion protein was administered on days 6–10 after tumor inoculation. We now examined the antitumor activity of mscIL-12.her2.IgG3 and rMuIL-12 at 1 μg/day and a higher dose (5 μg/day) for 5 days. These experiments were designed both to

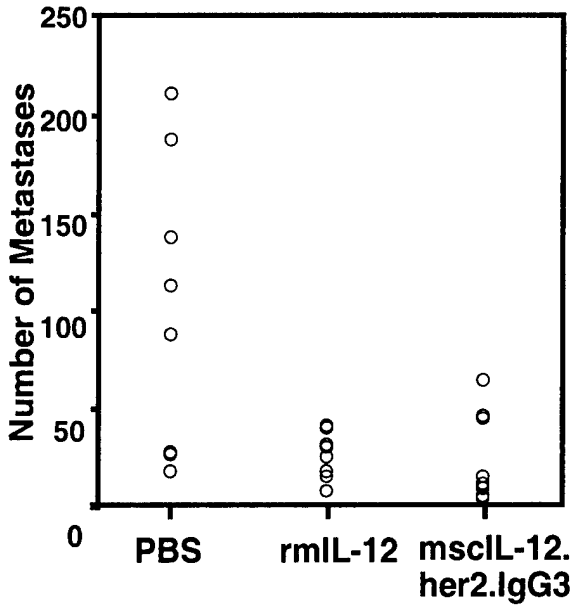

FIG. 1. Antitumor activity of rMuIL-12 and mscIL-12.her2.IgG3 in BALB/c mice bearing CT26-HER2/*neu* pulmonary metastases. BALB/c mice were injected with 5×10^4 CT26-HER2/*neu* cells i.v. on day 0. On days 3–7, the mice were treated with daily i.v. injection of PBS or PBS containing 1 μg IL-12 equivalent of mscIL-12.her2.IgG3 or 1 μg of rMuIL-12. On day 16, the mice were killed, the lungs were removed, and the metastases were counted.

compare the efficacy of the fusion protein with MuIL-12 and to determine if we could obtain complete tumor regression and if any treatment-limiting toxicity could be observed at the higher dose. We found that treatment of mice with rMuIL-12 or the equivalent IL-12 amount of mscIL-12.her2.IgG3 resulted in a potent inhibition of s.c. CT26-HER2/*neu* tumor growth when compared with the group injected with PBS, with more effective inhibition seen in the mice treated with the higher doses (Fig. 2). Although similarly significant ($p < 0.0001$) dose-dependent inhibition of tumor growth was seen for both treatment groups after day 15, significant antitumor activity was observed at an earlier time with mscIL-12.her2.IgG3 than with rMuIL-12. At days 7 and 9, mice injected with 5 μg rMuIL-12 equivalent of mscIL-12.her2.IgG3 showed a significant inhibition of tumor growth ($p = 0.007$ and $p = 0.004$, respectively) that was not observed in the group injected with 5 μg rMuIL-12 ($p = 0.5$ and $p = 0.4$, respectively). Similarly, mice injected with 1 μg rMuIL-12 equivalent of mscIL-12.her2.IgG3 showed a significant inhibition of tumor growth by days 11 and 13 ($p = 0.02$ for both days), whereas significant inhibition was not observed at that time in the group injected with 1 μg rMuIL-12 ($p = 0.16$ and $p = 0.18$, respectively). Complete tumor regression was not observed using either rMuIL-12 or mscIL-12.her2.IgG3 with these treatment regimens. Although the average volumes of the tumors in the two treatment groups were similar, as we had observed in the treatment of metastatic disease (Fig. 1), the response of mice to the treatment with rMuIL-12 was rather homogeneous, whereas animals treated with mscIL-12.her2.IgG3 split into two groups. One group showed very potent inhibition of s.c. tumor growth, and the other group

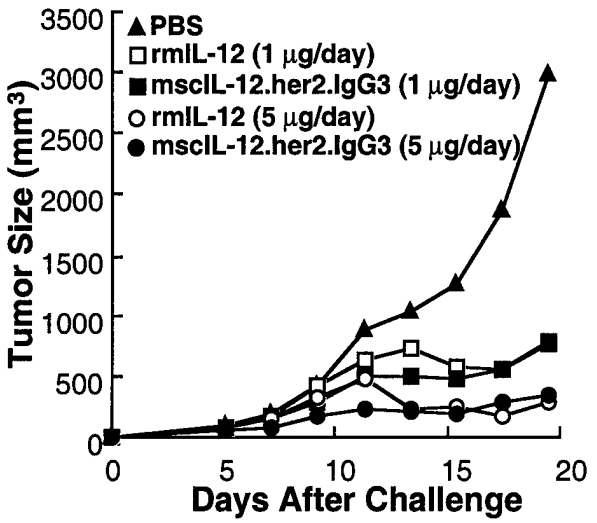

FIG. 2. Antitumor activity of different doses of rMuIL-12 and mscIL-12.her2.IgG3 in BALB/c mice bearing s.c. CT26-HER2/*neu* tumors. BALB/c mice were injected with 1×10^6 CT26-HER2/*neu* cells s.c. on day 0. On days 6–10, the mice were treated with daily i.v. injection of PBS or PBS containing 1 or 5 μg IL-12 equivalent of mscIL-12.her2.IgG3 or 1 or 5 μg rMuIL-12. The tumor size was monitored by caliper measurement every other day, and tumor volume was calculated.

had larger tumors than did the mice treated with rMuIL-12 (data not shown).

Examination of hematoxylin & eosin-stained histologic sections of tumors obtained from mice 19 days after injection of PBS (Fig. 3A), mscIL-12.her2.IgG3 (equivalent to 1 μg IL-12) (Fig. 3B), or 1 μg rMuIL-12 (Fig. 3C) showed that tumors from mice treated with PBS are highly cellular, whereas tumors from mice treated with mscIL-12.her2.IgG3 or rMuIL-12 show mononuclear cell infiltration and loss of structure. Although Figure 3 shows the tumor histology of only 1 mouse per each treatment group, similar results were observed in the other members of each treatment group (data not shown).

*Antitumor activity of rMuIL-12 and mscIL-12.her2.IgG3 in Rag2 knockout mice bearing s.c. CT26-HER2/*neu *tumors*

Since IL-12 antitumor activity has been attributed to T cells, we studied the activity of mscIL-12.her2.IgG3 in Rag2 knockout mice. These mice have a disruption of the *recombination activating gene 2 (Rag2)* and thus are unable to initiate V(D)J rearrangement and fail to generate mature B or T cells.[30] The antitumor activity of rMuIL-12 and mscIL-12.her2.IgG3 was markedly diminished (as compared with immunocompetent mice) but not completely abrogated in the Rag2 knockout mice (Fig. 4). In the case of mice injected with mscIL-12.her2.IgG3, statistically significant inhibition of tumor growth was seen on days 14, 16, 20, and 22 ($p \leq 0.04$) compared with the group treated with PBS. Although inhibition of tumor growth was observed in mice injected with rMuIL-12, it did not achieve statistical significance compared with the PBS treatment group. No mononuclear cell infiltration was observed in hematoxylin & eosin-stained sections of tumors obtained from Rag2 mice following injection of PBS, mscIL-12.her2.IgG3, or rMuIL-12 (data not shown).

*Antitumor activity of rMuIL-12 and mscIL-12.her2.IgG3 in SCID-beige mice bearing s.c. CT26-HER2/*neu *tumors*

To further identify the immune cells responsible for the antitumor activity of mscIL-12.her2.IgG3, we studied the efficacy of the fusion protein in SCID-beige mice. The SCID mutation causes a defect in V(D)J recombination, leading to a deficiency in B and T cells. The beige mutation causes impaired chemo-

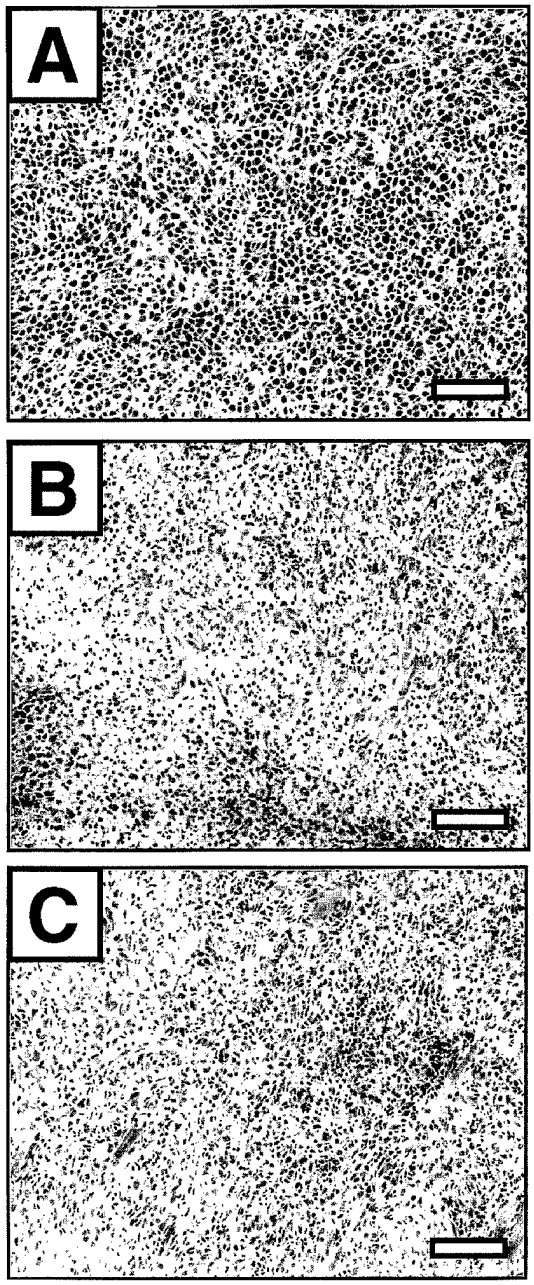

FIG. 3. Histologic sections of s.c. CT26-HER2/*neu* tumors from BALB/c mice. Twenty-day-old tumors from BALB/c mice were frozen and cut into 6-μm sections. Histologic sections of tumors from mice treated with PBS (**A**), mscIL-12.her2.IgG3 (**B**) and rMuIL-12 (**C**). The sections were stained with hematoxylin & eosin. Bar = 100 μm.

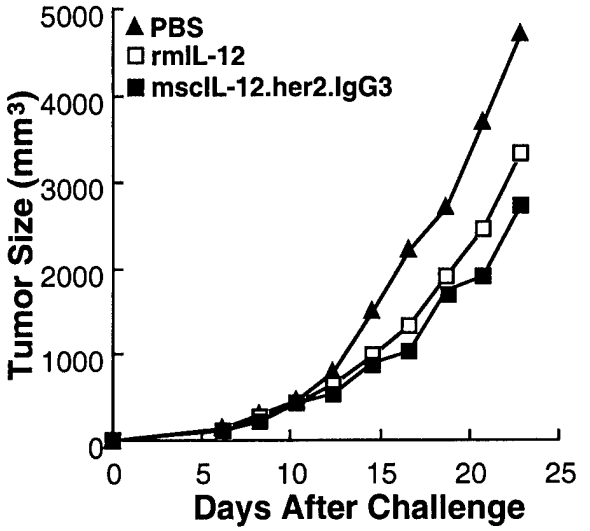

FIG. 4. Antitumor activity of rMuIL-12 and mscIL-12.her2.IgG3 in Rag2 knockout mice bearing s.c. CT26-HER2/*neu* tumors. Rag2 mice were inoculated s.c. with 1×10^6 CT26-HER2/*neu* tumor cells on day 0. On days 6–10, the mice were treated with daily i.v. injections of PBS or PBS containing 1 μg IL-12 equivalent of mscIL-12.her2.IgG3 or 1 μg rMuIL-12. The tumor size was monitored by caliper measurement every other day, and tumor volume was calculated.

taxis and motility of macrophages and a deficiency of NK cells.[31] Therefore, these mice should indicate if mscIL-12.her2.IgG3 and rMuIL-12 have any antitumor activity in mice deficient in T and NK cells and in macrophage activity. Figure 5 shows that the antitumor activity of rMuIL-12 and mscIL-12.her2.IgG3 was markedly diminished but not completely abrogated in the SCID-beige mice. Similar inhibition of tumor growth was seen in mice injected with mscIL-12.her2.IgG3 or rMuIL-2. By days 16 and 18, this inhibition was statistically significant ($p < 0.05$) compared with the group treated with PBS. Both mscIL-12.her2.IgG3 and rMuIL-12 demonstrated somewhat less antitumor activity in the SCID-beige mice than in the Rag2 knockout mice. The average tumor volume of mscIL-12.her2.IgG3-treated Rag2 knockout mice at day 20 was 1906 ± 581 mm^3, whereas in the SCID-beige mice, it was 2381 ± 804 mm^3. The Rag2 knockout and SCID-beige mice treated with PBS had similar average tumor volumes of 3695 ± 1513 mm^3 and 3772 ± 710 mm^3, respectively.

Antitumor activity of rMuIL-12 and mscIL-12.her2.IgG3 in BALB/c mice bearing s.c. CT26 tumors

Our studies have indicated that rMuIL-12 and mscIL-12.her2.IgG3 have similar antitumor activity against CT26-HER2/*neu*. To explore the contribution of antigen targeting to efficacy, we examined the effect of rMuIL-12 and mscIL-12.her2.IgG3 treatment on BALB/c mice bearing CT26 tumors that do not express HER2/*neu* (Fig. 6). In this system, both mscIL-12.her2.IgG3 and rMuIL-12 demonstrated antitumor activity, although the antitumor effect was not as great as that observed with the CT26-HER2/*neu* tumors. Whereas PBS-treated controls had average tumor volumes of 2500 mm^3, the rMuIL-12-treated mice had tumor volumes of 1845 mm^3 and 650 mm^3 and the mscIL-12.her2.IgG3-treated mice had tumors of 2387 mm^3 and 650 mm^3 for CT26 and CT26-HER/*neu*, respectively. On days 16 and 18, the inhibition of tumor growth was statistically significant ($p < 0.02$) for both mscIL-12.her2.IgG3 and rMuIL-12. Although rMuIL-12 appears to have a greater antitumor effect than mscIL-12.her2.IgG3, this difference is not statistically significant.

Distribution of CD3$^+$ and NK1.1$^+$ cells in the liver of tumor-bearing animals

To further understand the mechanism of the antitumor activity exhibited by mscIL-12.her2.IgG3, we examined mice bearing tumors at various times after treatment. On day 0, CT26-HER2/*neu* cells were injected s.c. into the right flank of BALB/c mice. The mice were treated with mscIL-12.her2.IgG3, rMuIL-12, or PBS for 5 days beginning on day 6. Mice were killed before the initiation of treatment (day 5) or 24 h (day 7), 4 days (day 10), and 7 days after treatment was initiated (day 13). Lymphocytes isolated from the livers of the mice were double-stained for NK cells (NK1.1) and T cells (CD3) and analyzed by flow cytometry. A representative flow cytometry profile of lymphocytes isolated from the liver at day 7 is shown in Figure 7A, with the regions gated representing CD3$^+$ (CD3$^+$/NK1.1$^-$), NK1.1$^+$ (CD3$^-$/NK1.1$^+$), and double-positive (CD3$^+$/NK1.1$^+$) cells. The distribution of the CD3$^+$, NK1.1$^+$, and double-positive cells for each treatment group is shown in Figure 7B.

Although all three treatment groups showed a similar decrease in the percentage of lymphocytes staining with CD3$^+$ or

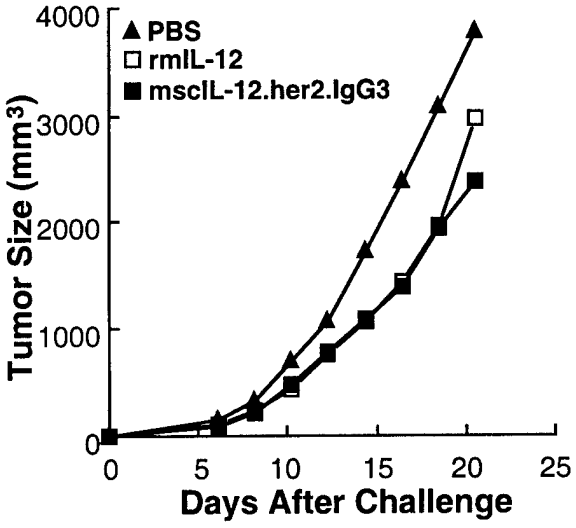

FIG. 5. Antitumor activity of rMuIL-12 and mscIL-12.her2.IgG3 in SCID-beige mice bearing s.c. CT26-HER2/*neu* tumors. SCID-beige mice were inoculated s.c. with 1×10^6 CT26-HER2/*neu* tumor cells on day 0. On days 6–10, the mice were treated with daily i.v. injections of PBS or PBS containing 1 μg IL-12 equivalent of mscIL-12.her2.IgG3 or 1 μg rMuIL-12. The tumor size was monitored by caliper measurement every other day, and tumor volume was calculated.

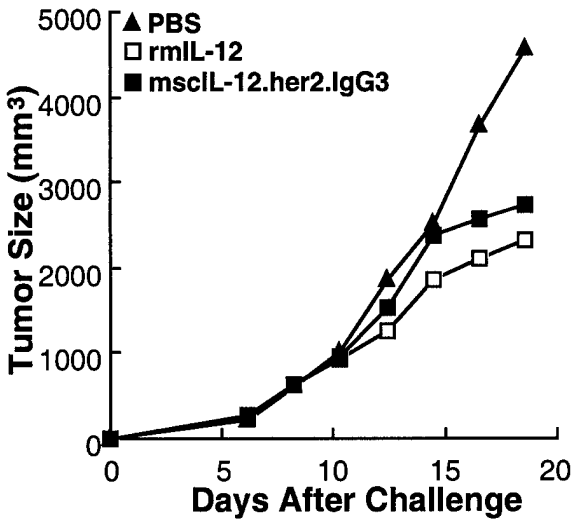

FIG. 6. Antitumor activity of rMuIL-12 and mscIL-12.her2.IgG3 in BALB/c mice bearing s.c. CT26 tumors. BALB/c mice were inoculated s.c. with 1×10^6 CT26 tumor cells on day 0. On days 6–10, the mice were treated with daily i.v. injections of PBS or PBS containing 1 μg IL-12 equivalent of mscIL-12.her2.IgG3 or 1 μg of MuIL-12. The tumor size was monitored by caliper measurement every other day, and tumor volume was calculated.

$NK1.1^+$ or both over time, there were differences among the groups in the percentage of $CD3^+$, $NK1.1^+$, and double-positive cells. Treatment with rMuIL-12 and mscIL-12.her2.IgG3 resulted in an increase in the percentage of $NK1.1^+$ cells that was seen clearly on days 10 and 13. This increase was seen in both $CD3^+/NK1.1^+$ and $CD3^-/NK1.1^+$ cells. These results demonstrate that both mscIL-12.her2.IgG3 and rMuIL-12 are able to increase comparably the number of $NK1.1^+$ cells in the liver. Especially noteworthy was the increase in $CD3^+/NK1.1^+$ cells seen on day 10 in the mscIL-12.her2.IgG3 treatment group.

*Isotype of the HER2/*neu*-specific antibody response*

The ability of IL-12 to switch the immune response to Th1 has been implicated in its antitumor activity. We previously reported that an anti-HER2/*neu* response was seen in our CT26-HER2/*neu* tumor model.[27] However, this response does not appear to affect tumor growth or to select for non-HER/*neu*-expressing tumor cells[27] or for loss of expression of MHC class I (M.L. Penichet et al., unpublished observations) in the tumor mass. To examine the isotypes of the HER2/*neu*-specific antibody response in BALB/c mice bearing CT26-HER2/*neu* tumors treated with PBS, rMuIL-12, and mscIL-12.her2.IgG3 (Fig. 1), serum collected on day 20 (approximately 14 days after treatment was initiated on day 6) from each treatment group was pooled and used in an anti-HER2/*neu* ELISA (Fig. 8). Mice treated with mscIL-12.her2.IgG3 or rMuIL-12 exhibited a decrease in IgG1 (Th2) HER2/*neu*-specific antibodies relative to PBS-treated controls. Variability is seen in the effects of rMuIL-12 and mscIL-12.her2.IgG3 on the levels of IgG2a (Th1) HER2/*neu*-specific antibodies, but in general we observed an increase in the IgG2a immune response in mice treated with free rMuIL-12 or IL-12 fused to IgG3 (mscIL-12.her2.IgG3). Similar results were obtained within a week of treatment on day 13 (data not shown).

Antiangiogenic activity

Our studies have demonstrated that mscIL-12.her2.IgG3 has antitumor activity that is attributable to T and NK cells. However, our data also suggest that additional mechanisms may be involved. To examine whether mscIL-12.her2.IgG3 has any antiangiogenic activity, tumor sections from various times during treatment were stained for PE-CAM, an adhesion molecule (CD31) on endothelial cells (Fig. 9A). Fields that spanned the entire area of the tumor section were photographed, grid was overlaid on the photographs, and vessel intersections were counted (Fig. 9B). A decrease in vessel count was seen 4 and 7 days after treatment with mscIL-12.her2.IgG3 and rMuIL-12 was initiated. These results demonstrate that both mscIL-12.her2.IgG3 and rMuIL-12 have antiangiogenic activity.

DISCUSSION

We previously described the construction of an IL-12-anti-HER-2/*neu* antibody fusion protein (mscIL-12.her2.IgG3) that retains antigen specificity and IL-12 bioactivity *in vitro* and demonstrates antitumor activity.[19] In these studies, we further characterize this antitumor activity in CT26-HER2/*neu* models[27] of s.c. and metastatic disease, and we describe the mechanism of this antitumor activity.

For these studies, we have used CT26 (also known as C-26 and colon tumor 26), a murine colon carcinoma (epithelial origin) syngeneic to BALB/c, that was induced by intrarectal injection of *N*-nitroso-*N*-methylurethan.[28] The CT26 tumor is a relevant model for testing immunotherapeutic approaches to cancer treatment because human cancers of epithelial origin are among the most difficult to treat by existing immunotherapies.[32,33] However, variable results have been reported following systemic IL-12 treatment of mice bearing CT26 tumors. Although there are reports that s.c. CT26 tumors growing in immunocompetent mice are refractory to systemic treatment with IL-12,[34,35] other authors, such as Tannenbaum et al.,[11] have reported that systemic treatment with IL-12 is highly effective. Our studies have shown that both CT26-HER2/*neu* and the parental CT26 tumors are sensitive to both rMuIL-12 and mscIL-12.her2.IgG3, highlighting the importance of using free rMuIL-12 as a control when evaluating fusion protein efficacy. However, we observed that CT26-HER2/*neu* responds much better to therapy with both rMuIL-12 and mscIL-12.her2.IgG3 than does the parental CT26 tumor. Therefore, it is possible that expression of the HER2/*neu* antigen altered the sensitivity of the CT26 cells to IL-12 or made the tumor cells more readily recognized by immune effector cells.

Although direct comparisons between the CT26 and CT26-HER2/*neu* results are not possible, our results are consistent with the study of Tannenbaum et al.,[11] which showed that CT26 responded to systemic treatment with IL-12. In that study, systemic treatment with IL-12 led to IFN-γ and IFN-inducible protein-10 (IP-10) production,[11] which could be detected as early as 4 h after IL-12 administration and became maximal at

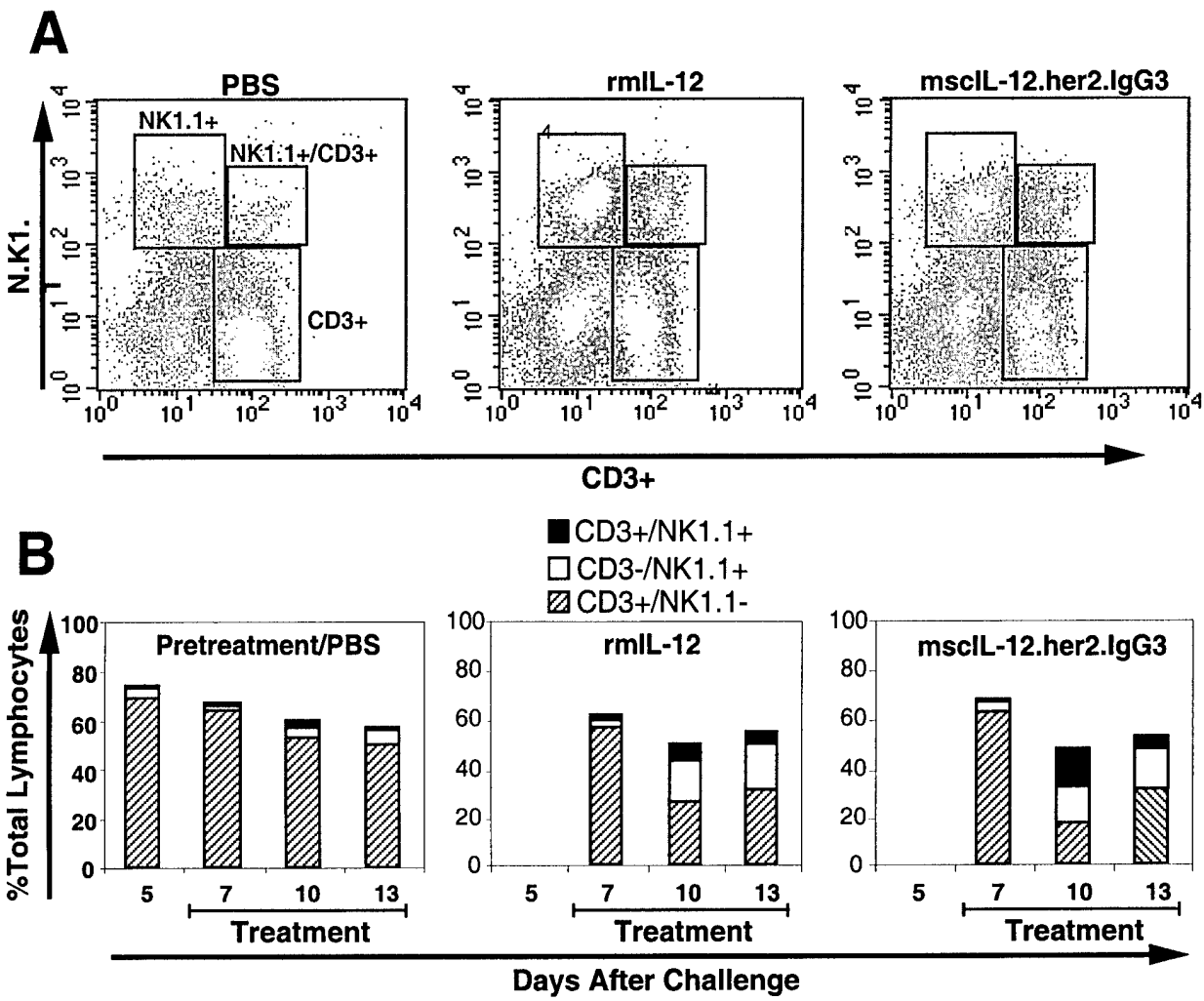

FIG. 7. Distribution of $CD3^+$ and $NK1.1^+$ cells in the liver of tumor-bearing animals. BALB/c mice bearing CT26-HER2/*neu* tumors were treated on days 6–10 with PBS, rMuIL-12, or mscIL-12.her2.IgG3. On days 5, 7, 10, and 13, mice were killed. Lymphocytes isolated from the livers (3 mice per group) were stained for CD3 and NK1.1 and analyzed by flow cytometry. (**A**) Flow cytometry profile of lymphocytes from liver stained for CD3 and NK1.1 at day 7. (**B**) Percentage of total lymphocytes from the animals that were $CD3^+/NK1.1^+$, $CD3^+/NK1.1^-$, or $CD3^-/NK1.1^+$.

approximately 6 days with continued IL-12 treatment. Further, immunohistologic analysis revealed infiltration with $CD8^+$ T cells and Mac-1^+ mononuclear cells (Mac-1 is found on NK cells and macrophages), but very low or negligible infiltration by $CD4^+$ T cells.[11] Tumor regression was associated with expression of perforin and granzyme B, consistent with the hypothesis that one mechanism by which cytolytic T and NK cells mediate cytolysis of tumor targets is by exocytosis of effector molecules stored in granules.[11]

Earlier studies had shown that treatment of SCID mice bearing pulmonary metastases of CT26 expressing human EpCAM with an anti-EpCAM antibody IL-12 fusion protein causes significant antitumor activity, although complete eradication of tumors was not achieved.[18] We now demonstrate using immunocompetent BALB/c instead of SCID mice that mscIL-12.her2.IgG3 is highly effective in inhibiting the number of CT26-HER2/*neu* pulmonary metastases. We also found that treatment of mice with free rMuIL-12 causes antitumor activity similar to mscIL-12.her2.IgG3 treatment. The control of free IL-12 was not used in the earlier studies.[18]

We have also found that treatment with rMuIL-12 or mscIL-12.her2.IgG3 results in a potent antitumor activity against s.c. CT26-HER2/*neu* tumors, with efficacy increasing at higher doses. This is consistent with previous reports showing that the antitumor effect of IL-12 is dose dependent and can be initiated against well-established tumors.[8] Tumor histology showed loss of structure and fragmentation associated with mononuclear cell infiltration, presumably T cells, as no infiltration is seen in Rag2-deficient mice following similar treatments. T cell infiltration is consistent with the ability of IL-12 to elicit a CTL immune response.[2,4,8] In fact, in patients receiving IL-12, tumor biopsy and immunohistochemistry studies revealed $CD4^+$ and $CD8^+$ T cell infiltration.[14,15]

The fact that similar antitumor activity is seen after treat-

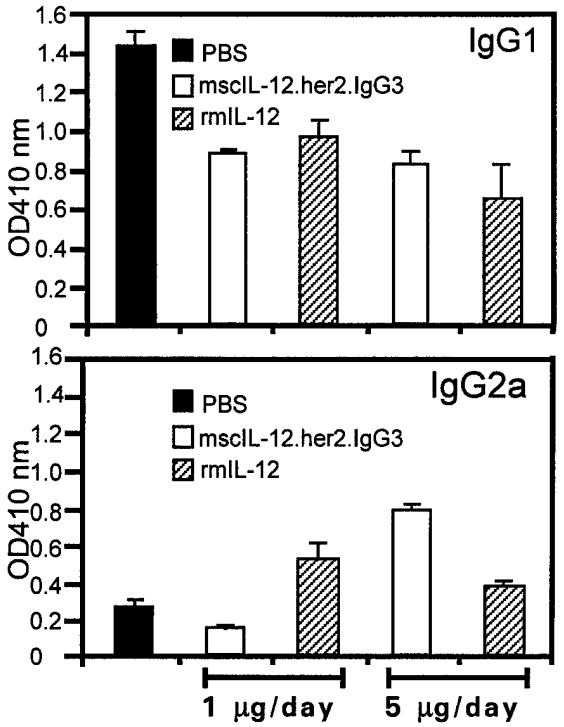

FIG. 8. Isotype of anti-HER/*neu* antibody response. Plates were coated with HER2/*neu* ECD^{HER2}. The serum from mice treated with PBS or 1 and 5 μg IL-12 equivalents per day of mscIL-12.her2.IgG3 or rMuIL-12 collected at day 20 was pooled, diluted 1:50, and added. Rat antimouse IgG1 and antimouse IgG2a antibodies were used as primary antibodies. Alkaline phosphatase-conjugated goat antirat secondary antibody followed by substrate were added, and the plates were read at 410 nm.

ment of both pulmonary and s.c. CT26-HER2/*neu* tumors with mscIL-12.her2.IgG3 and rMuIL-12 suggests that tumor targeting does not improve the antitumor response following treatment. However, mscIL-12.her2.IgG3 appeared more effective than rMuIL-12 at early times during treatment (Fig. 2). In addition, mscIL-12.her2.IgG3 was less effective than rMuIL-12 against CT26 that did not express the targeting antigen (although the difference did not reach statistical significance), suggesting that tumor targeting may play a role in the antitumor mechanism of mscIL-12.her2.IgG3. It is noteworthy that other investigators were unable to perform studies with IL-12 fusion proteins in immunocompetent mice because the fusion protein was too immunogenic.[18] Although we do see some efficacy of the fusion proteins in our studies, it is possible that the fused MuIL-12 acts as an adjuvant and increases the murine humoral immune response against human Ig sequences of the antibody-IL-12 fusion protein resulting in its neutralization and, as a consequence, in less effective antitumor activity. Similar enhancement of a murine antihuman IgG humoral immune response has been described for an anti-HER2/*neu* IgG3-granulocyte-macrophage colony-stimulating factor (GM-CSF) fusion protein.[36] This hypothesis may explain the observation that greater variation is seen in tumor size in mice treated with mscIL-12.her2.IgG3 than in those treated with rMuIL-12. It is possible that the mice with larger tumors mounted a more potent humoral immune response to mscIL-12.her2.IgG3. Further studies are required to investigate the relationship between the immunogenicity and efficacy of antibody-IL-12 fusion proteins. However, if the murine immune response to the human Ig sequences of the antibody-IL-12 fusion protein does limit efficacy in mouse models, this suggests that the antibody-IL-12 fusion protein will be much more effective in the treatment of patients, the ultimate goal of this fusion protein.

A role for T cells in the antitumor activity of IL-12 is supported by our experiments using Rag2 mice. In the Rag2 mice that lack B and T cells,[30] both IL-12 and mscIL-12.her2.IgG3 showed less potent antitumor activity than in immunocompetent mice, and there was a lack of mononuclear cell infiltration. It is also of interest that in these mice, which cannot mount an immune response to the antibody-IL-12 fusion protein, a statistically significant retardation in the tumor growth rate was found after treatment with mscIL-12.her2.IgG3 but not after treatment with free rMuIL-12. In these mice, NK or macrophage activation by IL-12 should be responsible for the antitumor activity.[8,9,37] In fact, the strong inhibition of CT26-EpCAM pulmonary metastases observed in SCID mice after treatment with the anti-EpCAM antibody-IL-12 fusion protein led the authors to suggest that in this model, the antibody-IL-12 fusion protein was a potent activator of NK cells.[18]

The further decrease in the antitumor activity of mscIL-12.her2.IgG3 that we observed in SCID-beige mice, which not only are T and B cell deficient but also have impaired chemotaxis and motility of macrophages and NK cell deficiency,[31] is consistent with macrophage or NK cell activation (or both) contributing to the antitumor activity of antibody-IL-12 fusion proteins. However, as there was some residual antitumor activity in the SCID-beige model, additional (noncellular) mechanisms must also contribute to the antitumor activity of mscIL-12.her2.IgG3. Indeed, we found that mscIL-12.her2.IgG3 and rMuIL-12 demonstrated significant antiangiogenic activity consistent with an increase in IFN-γ and subsequent increase in IP-10, an inhibitor of angiogenesis.[2,5,6]

We found that treatment with rMuIL-12 or mscIL-12.her2.IgG3 resulted in a change in the composition of the lymphocyte population present in the liver. We observed an increase in the percentage of both $CD3^-/NK1.1^+$ and double-positive ($CD3^+/NK1.1^+$) cells. This increase persisted throughout the course of the experiment (Fig. 7). In contrast, Fogler et al.[38] found that IL-12 administration (0.5 μg/day i.p. for 7 days) resulted in an increase in hepatic $NK1.1^+$ cells 24 h after IL-12 administration was initiated, but this increase was not sustained. Instead, $CD3^+$ cells gradually increased during the time that IL-12 was administered. The differences between what we and Folger et al. observed might be explained by the different dosing regimens. IL-12 stimulates the production of IL-2. High doses of IL-2 are known to stimulate NK cell-mediated antitumor activity, whereas low doses stimulate a T cell-mediated effect.[39] Different dosing regimens and routes of administration of IL-12 may lead to different levels of IL-2 induction. The use of different mouse strains may also contribute to the differences, as Fogler et al.[38] used C57BL/6 mice, and we used BALB/c mice. C57BL/6 mice produce a Th1 response to infection with *Leishmania major*, and BALB/c mice generate

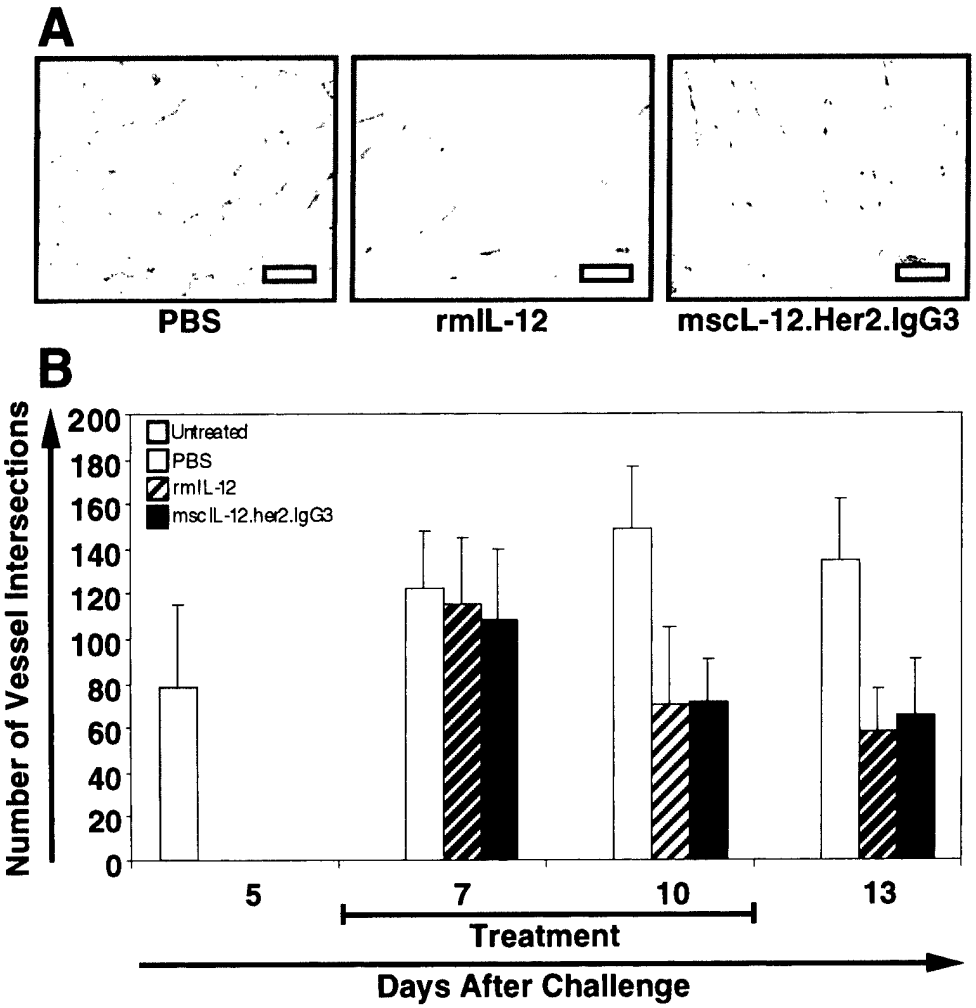

FIG. 9. Antiangiogenic activity. BALB/c mice bearing s.c. CT26-HER2/*neu* tumors were treated on days 6–10 with PBS, rMuIL-12, or mscIL-12.her2.IgG3. On days 5, 7, 10, and 13, mice (3 mice per group) were killed. Tumor sections were stained for PE-CAM (a cell adhesion molecule on endothelial cells) and counterstained with nuclear fast red. (**A**) Tumor sections at day 13 after tumor challenge (day 7 after the initiation of treatment). Bar = 100 μm. (**B**) Photographs of the sections (at least three fields per tumor section, average five) were taken, the photographs were overlaid with a 5 × 5-μm grid, and vessel intersections were counted. The number of intersections per view was averaged for all tumors within the same treatment group at the same time point, and standard deviations were calculated and plotted.

a Th2 response.[40] Hashimoto et al.[41] had previously demonstrated that NK1.1$^+$/CD3$^+$ cells are induced in the liver by IL-12 and that these cells demonstrate MHC-nonrestricted cytotoxic activity. Thus, the increase in NK and NK1.1$^+$/CD3$^+$ cells after treatment with mscIL-12.her2.IgG3 may contribute to its antitumor activity.

In conclusion, it would appear that the antitumor activity of mscIL-12.her2.IgG3 in a CT26-HER2/*neu* model is highly complex and involves the activity of T cells, NK cells, or macrophages, as well as antiangiogenic effects. Therefore, in spite of the genetic modification of IL-12 in the fusion protein, it maintains an activity profile similar to that of IL-12. The potent antitumor activity of the anti-HER2/*neu*-IL-12 fusion protein suggests that it may be of clinical utility in the treatment of patients with tumors expressing HER2/*neu*.

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Protein vaccination with the HER2/*neu* extracellular domain plus anti-HER2/*neu* antibody-cytokine fusion proteins induces a protective anti-HER2/*neu* immune response in mice.

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1. Abstract

Previously protein vaccines consisting of the extracellular domain of HER2/*neu* (ECD^{HER2}) were shown to elicit an immune response that does not provide protection against tumors expressing HER2/*neu*. Here we showed that when mice were vaccinated with a mixture of human ECD^{HER2} and anti-human HER2/*neu* IL-12, IL-2 or GM-CSF fusion proteins, significant retardation of the growth of a syngeneic carcinoma expressing rat HER2/*neu*, TUBO, with long-term survivors was observed. Immune sera inhibited the *in vitro* growth of SK-BR-3, a human breast cancer overexpressing HER2/*neu*. Transfer of immune sera into mice challenged with TUBO also led to partial inhibition of tumor growth. Splenocytes from mice vaccinated with ECD^{HER2} plus IgG3-(GM-CSF) incubated with ECD^{HER2} demonstrated significant proliferation and IFN-γ secretion. Taken together these results suggest that vaccines including ECD^{HER2} and Ab-cytokine fusion proteins may be used to elicit both humoral and cell-mediated responses against HER2/*neu*.

Keywords: Antibodies; Cytokines; Immunotherapy

Running Title: anti-human HER2/*neu* Ab-cytokine fusion proteins for cancer therapy

2. Introduction

HER2/*neu* is a 185 kDa protein with extracellular (ECD^{HER2}), transmembrane, and intracellular domains (ICD). HER2/*neu* (also known as p185 or c-erb-B2) is a growth factor receptor which can transduce cell signaling and play a role in cell differentiation, adhesion and motility [1]. HER2/*neu* is overexpressed in 20%-40% of breast cancers [2] as well as in ovarian, endometrial, gastric, bladder, prostate [3] and lung cancer [1] with overexpression sometimes 100 fold higher than in normal tissues as a result of HER2/*neu* gene amplification [4]. Studies suggest that the overexpression of HER2/*neu* plays a direct role in the pathogenesis and aggressiveness of tumors [1] and is associated with a poor clinical outcome in patients with newly diagnosed primary breast cancer [2]. At present, treatment of patients with advanced HER2/*neu*-expressing breast cancer with the anti-HER2/*neu* antibody (Ab), Trastuzumab (Herceptin, Genentech, San Francisco, CA), can lead to an objective anti-tumor response [1]. Chemotherapy can synergize with Trastuzumab to enhance its anti-tumor activity [1]. However, a positive response is observed in only a subset of patients [1] and additional modalities designed to improve clinical outcome are still needed.

HER2/*neu* is an attractive target for active immunotherapy due to its low expression in normal tissues and its overexpression in many different types of cancers [1]. It is anticipated that a vaccine specific for HER2/*neu* would have wide application in the treatment and /or prevention of many different human malignancies [5]. Indeed, DNA-based vaccines were able to induce protective immunity against rat HER2/*neu* expressing tumors in rat HER2/*neu* transgenic mice [6-8]. Peptide-based vaccines were

also able to "break" tolerance and generate anti-tumor activity in animal models [9, 10]. Although rats immunized with rat HER2/*neu* in incomplete Freund's adjuvant showed no rat HER2/*neu* specific response [10], immunization of rats with the human HER2/*neu* ECD (ECD^{HER2}) protein did elicit an immune response to rat HER2/*neu* [11], suggesting that foreign proteins with high homology to "self" tumor antigens, may be effective in generating a response to "self" tumor antigens [12]. However the resulting immune response did not confer protection against a rat HER2/*neu* expressing tumor [11]. Immunocompetent mice also did not reject syngeneic tumors expressing a xenogenic and immunogenic human HER2/*neu* protein [13-15]. Thus breaking tolerance to a self tumor antigen may not be sufficient to confer tumor protection.

We have previously shown that anti-HER2/*neu* Ab (human IgG3) genetically fused to cytokines IL-2, IL-12 or GM-CSF (Ab-cytokine fusion protiens) [16-18] induces anti-tumor activity when injected into mice bearing HER2/*neu* expressing tumor cells [16-18]. These and other studies [19, 20] suggest that the immunomodulatory effects of these fused cytokines can potentiate an anti-tumor immune response.

In contrast to our earlier studies, the present study uses Ab-cytokine fusion proteins with ECD^{HER2} vaccination in an attempt to induce an immune response that will protect against subsequent tumor challenge. Female BALB/c mice were vaccinated with the human ECD^{HER2} protein or with ECD^{HER2} in an immune complex with Ab-cytokine fusion proteins. When vaccinated mice were challenged with a transplantable carcinoma, TUBO, overexpressing rat HER2/*neu* [6], an enhancement of anti-tumor activity was seen in mice immunized with the immune complexes, whereas mice immunized with soluble ECD^{HER2} showed only modest anti-tumor immunity compared to the control

group. These results are in contrast to previous studies in which ECD^{HER2} vaccination using enhancers such as montanide 720 and complete Freund's adjuvant did not elicit anti-tumor responses in animals challenged with HER2/*neu* expressing tumors [11, 13], and suggest that it may be possible to use ECD^{HER2} vaccination in combination with Ab-cytokine fusion proteins in the treatment of patients with HER2/*neu* expressing cancers.

3. Materials and Methods

3.1 Mice

Female BALB/c mice 10-12 weeks of age obtained from Taconic Farms, Inc. (Germantown, NY) were used. All experiments were performed according to National Institutes of Health (NIH) (Bethesda, MD) Guide for the Care and Use of Laboratory Animals.

3.2 Cell lines

TUBO, a cloned cell line overexpressing the rat HER2/*neu* protein, was established from a lobular carcinoma that spontaneously arose in a female BALB/c mouse transgenic for the transforming rat *neu* oncogene driven by the mouse mammary tumor virus promoter [6]. TUBO cells grow progressively in normal BALB/c mice and give rise to lobular carcinoma, histologically similar to that seen in BALB-neuT-transgenic mice [6]. TUBO cells were cultured in Dubecco's Modified Eagle Medium, (DMEM), supplemented with glutamax, glucose, 25 mM Hepes buffer, pyridoxine-HCl (GibcoBRL, Life Technologies,

Rockville, MD) with 20% fetal bovine serum (Atlas biologicals, Fort Collins, CO). SK-BR-3 is a human breast cancer cell line overexpressing the human HER2/*neu* protein (ATCC, Rockville, MD). SK-BR-3 cells were cultured in Iscoves Modified Dubecco's Medium, IMDM (Irvine Scientific Inc, Irvine, CA), supplemented with L-glutamine, penicillin, and streptomycin with 5% bovine calf serum (Atlanta biologicals, Norcross, GA).

3.3 Ab-cytokine fusion proteins and ECD^{HER2}

The construction, purification and analysis of biological activities of IgG3, IgG3-(GM-CSF), IgG3-(IL-2) and IgG3-(IL-12) cytokine fusion proteins were described previously. IgG3 and Ab-cytokine fusion proteins consist of the human IgG3 containing the same variable region as the recombinant anti-HER2/*neu*, Herceptin [16-18].

Dr. James D. Marks (University of California at San Francisco, San Francisco, CA) provided us with BHK/erbB2, a cell line that secretes soluble human ECD^{HER2}. Soluble ECD^{HER2} was purified from BHK/erbB2 culture supernatants using affinity chromatography with anti-HER2/*neu* IgG3 immobilized on Sepharose 4B (CNBr-activated Sepharose 4B, Amersham Pharmacia Biotech, Upsala, Sweden). All purified proteins were dialyzed against dialysis buffer (50 mM Tris base, 150 mM NaCl in deionized water at pH 7.8) and the concentrations determined by bicinchoninic acid based protein assay (BCA Protein Assay, Pierce Chemical Co., Rockford, IL). Prior to use, proteins were analyzed by SDS-PAGE and Coomassie blue stained to assess purity, size and integrity.

3.4 Vaccination of mice and challenge with TUBO

Two groups of eight mice were injected s.c. in their right flanks on day 0 and again (boosting) on day 35 (week 5) with either 8 μg of ECD^{HER2} alone, 8 μg of ECD^{HER2} plus 14 μg of IgG3, 8 μg of ECD^{HER2} plus 16 μg of IgG3-(GM-CSF), 8 μg of ECD^{HER2} plus 16 μg of IgG3-(IL-2), or 8μg of ECD^{HER2} plus 27 μg of IgG3-(IL-12). Ab or Ab-cytokine fusion proteins were mixed with ECD^{HER2} at a 1:1 molar ratio, at a concentration to allow the injection of 150 μl per mouse, and allowed to sit at 4 ^{o}C overnight prior to injection. Mice injected with diluent (PBS) served as a control group. Three weeks after the boosting, one set of vaccinated mice was challenged in the left flank with 10^6 TUBO cells in 150 μl Hank's balanced salt solution, HBSS, (GIBCO BRL, Life Technologies, Rockville, MD). One out of the eight mice vaccinated with ECD^{HER2} plus IgG3-(IL-2) died prior to a challenge with TUBO cells from a cause unrelated to the vaccination. Tumor growth was monitored and measured with a caliper beginning 7 days after the tumor challenge. Mice with tumors 1.5 cm in diameter or greater were euthanized. On the same day the vaccinated mice were challenged with TUBO, blood (used in serological studies and passive transfer of immunity) and splenocytes were collected from the other group of unchallenged vaccinated mice and processed and used in additional studies described below.

3.5 Characterization of murine Ab response to ECD^{HER2}

Sera obtained from mice 2 days prior to a challenge with TUBO or from unchallenged vaccinated mice were analyzed by ELISA for Abs to ECD^{HER2} using 96-well microtiter plates coated with 50 μl of ECD^{HER2} at a concentration of 1 μg/ml. The plates were washed and blocked with 3% bovine serum albumin (BSA) (Sigma Chemical, St. Louis, MO) in PBS. After washing, dilutions of sera in PBS containing 1% BSA were added to the wells and incubated overnight at 4 ^{o}C. Bound IgG was detected by incubating for 1 hr

at 37 ^{o}C with AP-labeled rabbit anti-mouse IgG (Zymed, San Francisco, CA). After washing, *p*-nitrophenyl phosphate disodium dissolved in diethanolamine buffer (Sigma Chemical, St. Louis, MO) was added for 2 hrs and the plates were read at 410 nm. Sera from naïve mice of the same age were used as a negative control. All ELISAs were made in duplicate using an internal positive control curve for each plate. Anti-ECD^{HER2} IgG1, IgG2a and IgG3 responses were analyzed by ELISA using 96-well microtiter plates prepared as described above with AP-labeled rat anti-mouse IgG1, IgG2a (Zymed, San Francisco, CA) or AP-labeled goat anti-mouse IgG3 (Southern Biotechnology Associates, Inc., Birmingham, AL) used as detecting agents.

3.6 SK-BR-3 *in vitro* proliferation assay

2 x 10^4 SK-BR-3 cells in 100 μl of IMDM supplemented with L-glutamine, penicillin, streptomycin and 5% bovine calf serum were added to each well of a 96-well round bottom tissue culture plate. Pooled sera from each regimen were depleted of complement by incubation at 56 ^{o}C for 30 minutes, diluted in the same medium to give a final working dilution of 1:100 and 1:300, and 100 μl added to SK-BR-3 cells which were then incubated for 6 days in a 5% CO_2, 37 ^{o}C incubator. Wells were pulsed with ^{3}H-thymidine (ICN, Costa Mesa, CA) at a final concentration of 5 μCi/ml 12 hrs prior to the end of the incubation period, the cells harvested and passed through a glass-fiber filter (Wallac Oy, Turku, Finland) using a Micro Cell Harvester (Skatron, Norway). ^{3}H-thymidine incorporation into DNA by actively growing cells was measured with a 1205 Betaplate Liquid Scintillation Counter (Wallac Oy, Turku, Finland). All assays were done in triplicate. Data are presented as ^{3}H-thymidine (CPM) incorporation by SK-BR-3 cells

after incubation with immune sera. IgG3, containing the same variable region as Herceptin, is effective in inhibiting the growth of SK-BR-3, *in vitro*, and was used as a positive control.

3.7 Transfer of immune sera

Mice were randomized and distributed into 6 mice per group. At day -1, naïve mice received an i.v. injection of 175 μl (the maximum volume available per mouse), of pooled sera collected from the unchallenged vaccinated mice. On day 0, 10^6 TUBO cells in 150 μl of HBSS were injected in the right flank. An untreated group of the same age was also challenged with TUBO cells.

3.8 Mouse splenocyte isolation, stimulation with soluble ECD^{HER2} and cytokine quantification

Spleens from the unchallenged vaccinated mice were removed, pooled and teased with two frosted specimen slides, using aseptic techniques. Released splenocytes were passed through a 100 μm cell strainer (Becton Dickinson Labware, Franklin Lanes, NJ) to remove large debris and RBCs were lysed in 0.85% ammonium chloride made in deionized water. 5 x 10^6 splenocytes/ml/well were added into wells of a 24-well tissue culture plate in RPMI 1640 (GibcoBRL, Life Technologies, Rockville, MD) supplemented with 50 IU/ml of murine IL-2 (PeproTech. Inc., Rocky Hill, NJ), 10% fetal bovine serum and 1 μg/ml of soluble ECD^{HER2} protein, and incubated in a 5% CO_2, 37 ^oC incubator. After 84 hrs, 5 μCi of ^{3}H-thymidine was added to each well and incubation continued for 12 hrs. Cells from a single well of a 24-well tissue culture plate were

transferred to four wells of a 96-well round bottom tissue culture plate, harvested and ^{3}H-thymidine incorporation into DNA was measured as described above. Data are expressed as a stimulation index (SI) which is defined as the mean ^{3}H CPM of the experimental wells divided by the mean ^{3}H CPM of the control wells (splenocytes of mice vaccinated with PBS).

To determine the level of secreted IFN-γ or IL-4, supernatants from a single well of a 24-well tissue culture plate were removed after 36 and 84 hrs of stimulation and added, in duplicate, into 96-well microtiter plates pre-coated with anti-IFN-γ or anti-IL-4 capture Ab (PharMingen, San Diego, CA). Supernatants were diluted serially 1:2 and allowed to sit overnight at 4 ^{o}C. The following day, the plates were washed, detecting AP-labeled Ab (PharMingen, San Diego, CA) added and the plates were allowed to sit at 37 ^{o}C for 1 hr. After washing, *p*-nitrophenyl phosphate disodium dissolved in diethanolamine buffer was added to the wells and the plates were read at 410 nm. Quantitation of results was performed using an IFN-γ (PharMingen, San Diego, CA) or IL-4 (PeproTech, Inc., Rocky Hill, NJ) standard curve generated in each plate.

3.9 Statistical analysis

All statistical analysis were made using the Mann-Whitney Rank Test, except for the survival curve for which the Trend Peto-Peto-Wilcoxon Test was used. For all cases, results were regarded significant if p values were ≤ 0.05.

4. Results

4.1 ECD^{HER2} vaccination with the Ab-cytokine fusion proteins enhances the anti-tumor immune response

To determine if Ab-cytokine fusion proteins would enhance the anti-tumor response obtained with HER2/*neu* ECD vaccination, female BALB/c mice were injected s.c. on week 0 and week 5 with either PBS, ECD^{HER2} alone, ECD^{HER2} plus IgG3 or ECD^{HER2} plus either IgG3-(GM-CSF), IgG3-(IL-2) or IgG3-(IL-12) as described above. Eight weeks after the intial injection, 10^6 TUBO cells were injected s.c. into the left flank of vaccinated mice. Tumors grew progressively in all PBS, ECD^{HER2} alone and ECD^{HER2} plus IgG3 treated mice with no mice free of tumors. In contrast, at 19 days, five mice vaccinated with ECD^{HER2} plus IgG3-(IL-2), and three mice vaccinated with ECD^{HER2} plus IgG3-(IL-12) or ECD^{HER2} plus IgG3-(GM-CSF) remained tumor free (Fig 1A).

Mice bearing tumors greater than 1.5 cm in diameter at the time of inspection were euthanized and considered to have not survived the challenge with TUBO. A survival curve, taking this into consideration, shows the superiority of vaccination regimens in which ECD^{HER2} is combined with Ab-cytokine fusion proteins ($p< 0.05$, compared to ECD^{HER2} alone or ECD^{HER2} plus IgG3 vaccinated mice) (Fig. 1B). No significant difference was observed between mice vaccinated with ECD^{HER2} plus IgG3 and ECD^{HER2} alone ($p= 0.20$). 36 days after challenge only one mouse of the 24 treated with PBS, ECD^{HER2} alone or ECD^{HER2} plus IgG3 remained alive. In contrast, 17 of the 23 mice treated with ECD^{HER2} plus Ab-cytokine fusion proteins remained alive. 110 days post-challenge, one out of eight mice vaccinated with ECD^{HER2} plus IgG3-(GM-CSF) or

ECD^{HER2} plus IgG3-(IL-2) and two out of eight vaccinated with ECD^{HER2} plus IgG3-(IL-12) remained tumor free (Fig. 1B). These results suggest significant protection against tumors expressing HER2/*neu* is achieved only when ECD^{HER2} vaccination is combined with the Ab-cytokine fusion proteins.

4.2 ECD^{HER2} vaccination with the Ab-cytokine fusion proteins affects both the quality and magnitude of the Ab response to ECD^{HER2}

To examine the anti-ECD^{HER2} response in vaccinated mice blood samples were collected from individual mice two days prior to tumor challenge. ECD^{HER2} plus IgG3-(GM-CSF) and ECD^{HER2} plus IgG3-(IL-2) vaccinated mice showed a higher anti-ECD^{HER2} IgG1 response compared to mice vaccinated with ECD^{HER2} plus IgG3-(IL-12) (p <0.001, Table Ia). Interestingly, mice vaccinated with ECD^{HER2} plus IgG3-(IL-12) showed a smaller IgG1 response (p < 0.07 or 0.01) compared to mice vaccinated with ECD^{HER2} or ECD^{HER2} plus IgG3, respectively (Table I a). In contrast, the IgG2a anti-ECD^{HER2} response was markedly increased in ECD^{HER2} plus IgG3-(IL-12) vaccinated mice (p <0.005), compared to all groups. A modest IgG2a response was seen in mice vaccinated with ECD^{HER2} plus IgG3-(IL-2) with lower levels in ECD^{HER2} plus IgG3-(GM-CSF); little to no IgG2a anti-ECD^{HER2} was detected in mice vaccinated with ECD^{HER2} alone or ECD^{HER2} plus IgG3 (Table I b). Thus, theses results suggest that while a comparable level of anti-tumor activity is seen in mice vaccinated with ECD^{HER2} plus any of the Ab-cytokine fusion proteins (Fig. 1), the quality and magnitude of the elicited anti-ECD^{HER2} response differ.

4.3 Immune sera inhibits the growth of SK-BR-3 in vitro

SK-BR-3 is a human breast cancer cell line overexpressing HER2/*neu* whose growth *in vitro* is inhibited by the anti-HER2/*neu* Ab 4D5 [21]. To determine if immune sera of vaccinated mice can inhibit the growth of SK-BR-3, pooled sera from vaccinated mice were added to the cells and cell proliferation was determined by ^{3}H thymidine incorporation as described above. Immune sera significantly inhibited the growth of SK-BR-3 (Fig. 2A,B) with the level of inhibition correlated with the level of anti-ECD^{HER2} IgG (Fig. 2C).

4.4 Anti-tumor activity can be transferred with immune sera

To determine if anti-tumor activity could be transferred by immune sera, mice were injected i.v. with pooled immune sera and then challenged s.c. the next day with 10^{6} TUBO cells. No apparent anti-tumor activity was observed with sera from mice vaccinated with PBS, ECD^{HER2} alone or ECD^{HER2} plus IgG3 (Table II). Although the tumors in mice injected with sera from mice vaccinated with ECD^{HER2} plus the Ab-cytokine fusion proteins were smaller when compared to the untreated mice, only at day 13 was that difference statistically significant in those mice injected with sera from mice vaccinated with ECD^{HER2} plus IgG3-(GM-CSF) (p=0.03, compared to the untreated mice). Mice injected with sera from mice vaccinated with ECD^{HER2} plus IgG3-(IL-2) showed significantly smaller tumors only at days 16 and 19 (p=0.03 and p=0.05 compared to the untreated mice, respectively). Mice injected with sera from mice

vaccinated with ECD^{HER2} plus IgG3-(IL-12) showed significantly smaller tumors at days 13, 16, 19 and 21 ($p \leq 0.05$ as compared to untreated mice) (Table II).

The transferred immune sera were analyzed for their relative levels of IgG1, IgG2a and IgG3 anti-ECD^{HER2}. Pooled sera from mice vaccinated with ECD^{HER2} plus IgG3-(GM-CSF) and ECD^{HER2} plus IgG3-(IL-2) showed higher levels of anti-ECD^{HER2} IgG1 compared to pooled sera from mice vaccinated with ECD^{HER2} plus IgG3-(IL-12), ECD^{HER2} plus IgG3 or ECD^{HER2} alone (Fig. 3A). In contrast, the anti-ECD^{HER2} IgG2a response was markedly higher in pooled sera from mice vaccinated with ECD^{HER2} plus IgG3-(IL-12), while a modest response was seen in mice vaccinated with ECD^{HER2} plus IgG3-(IL-2) or ECD^{HER2} alone (Fig. 3B). Analysis of serum from individual mice revealed that one out of eight mice vaccinated with ECD^{HER2} showed a strong anti-ECD^{HER2} IgG2a response, while little or no response was detected in the other seven mice (data not shown). Anti-ECD^{HER2} IgG3 levels were highest in mice vaccinated with ECD^{HER2} plus IgG3-(IL-12), intermediate in mice vaccinated with ECD^{HER2} plus IgG3-(IL-2) or ECD^{HER2} plus IgG3-(GM-CSF) and modest in mice immunized with ECD^{HER2} plus IgG3 or ECD^{HER2} alone (Fig. 3C). The pooled sera from mice immunized with ECD^{HER2} plus IgG3-(IL-12) was the most protective and had elevated levels of IgG2a and IgG3 but not IgG1 anti-ECD^{HER2}. Therefore, the ability of immune sera to transfer protection does not seem to correlate with the magnitude but rather the quality of the anti-ECD^{HER2} response.

4.5 In vitro stimulation of splenocytes, from vaccinated mice, by ECD^{HER2} protein reveals ECD^{HER2} vaccination with the Ab-cytokine fusion proteins affects the magnitude of cell-mediated immune response

To see if there was evidence for a cell-mediated immune response in vaccinated mice, the ability of splenocytes to proliferate following incubation with soluble ECD^{HER2} protein *in vitro* was assessed. Proliferation was measured by ^{3}H-thymidine incorporation into DNA. After 48 hrs of incubation, significant proliferation was detected in splenocytes from mice vaccinated with ECD^{HER2} plus IgG3-(GM-CSF) with less proliferation seen with splenocytes from mice vaccinated with ECD^{HER2} plus IgG3-(IL-2). Very modest stimulation was observed when the splenocytes were from mice vaccinated with ECD^{HER2} plus IgG3-(IL-12), ECD^{HER2} plus IgG3 or ECD^{HER2} alone (Fig. 4A). Similar results were observed after 96 hrs of incubation with the soluble ECD^{HER2} protein (Fig. 4B). Thus the Ab-fusion proteins differ in their affects on the cellular immune response to ECD^{HER2}. The cellular response observed *in vitro* does not correlate with the level of anti-tumor activity observed *in vivo* following tumor challenge.

4.6 Cytokine production of stimulated splenocytes

The level of IFN-γ and IL-4 cytokines characteristic of T_H1 or T_H2 responses, respectively [22], were determined in the supernatants of splenocytes incubated with the soluble ECD^{HER2} protein. After a stimulation period of 36 hrs, increased IFN-γ production was detected in the supernatants of splenocytes from vaccinated mice compared to the PBS control with the level: ECD^{HER2} plus IgG3-(GM-CSF) > ECD^{HER2} plus IgG3-(IL-2) > ECD^{HER2} plus IgG3-(IL-12) > ECD^{HER2} plus IgG3 > ECD^{HER2} alone (Fig. 5A). No IFN-γ could be detected when splenocytes from mice treated with PBS were used. After 84 hrs of stimulation, significant further accumulation of IFN-γ (1,600 pg/ml) was detected in the supernatant of splenocytes from mice vaccinated with ECD^{HER2} plus IgG3-(GM-CSF)

(Fig 5B). Modest accumulation of IFN-γ was observed in the supernatants of splenocytes of mice vaccinated with ECD^{HER2} plus IgG3-(IL-12), ECD^{HER2} plus IgG3-(IL-2), ECD^{HER2} plus IgG3, or ECD^{HER2} alone between 36 and 84 hrs (Fig 5B) . After 36 hrs the IL-4 level in all supernatants was below the sensitivity of the assay (<30 pg/ml, data not shown). After 84 hrs low levels of IL-4 (30 pg/ml) could be detected only in the supernatant of splenocytes from mice vaccinated with ECD^{HER2} plus IgG3-(GM-CSF). Hence, the robust accumulation of IFN-γ suggests that the observed cellular response (Fig. 4) is mainly T_H1 mediated.

5. Discussion

Although humoral and cellular immunity against the tumor-associated antigen, HER2/*neu*, have been shown to be present in patients with HER2/*neu* bearing malignancies [12, 21, 23], this immunity is clearly not sufficient to provide patients with protection. While it is anticipated that augmenting pre-existing immunity may have therapeutic effects [12], recent clinical trials aimed at boosting immunity to HER2/*neu* using peptide or peptide-pulsed dendritic cell-based vaccines, have yet to show clinical efficacy [24-26]. Thus, vaccine modalities targeting the HER2/*neu* protein designed not only to break immune tolerance and boost pre-existing immunity, but to also generate an immune response that can eradicate the cancer, are still needed.

Previous studies with HER2/*neu* ECD vaccination using conventional chemical adjuvants did not elicit anti-tumor responses in animals challenged with HER2/*neu* expressing tumors [11, 13]. In the present study we have shown that a mixture of HER2/*neu* ECD and Ab-cytokine fusion proteins containing genetically fused IL-12, IL-2 and GM-CSF provides more efficient protection than ECD^{HER2} alone, suggesting that anti-HER2/*neu* Ab-cytokine fusion proteins may be used to potentiate the anti-tumor response obtained with ECD^{HER2} protein vaccination.

The quality and magnitude of the anti-ECD^{HER2} Ab response depended on the immunization regimen. The most vigorous anti-ECD^{HER2} IgG1 response was in mice vaccinated with IgG3-(GM-CSF) and ECD^{HER2} plus IgG3-(IL-2) whereas mice vaccinated with ECD^{HER2} plus IgG3-(IL-12) showed the greatest enhancement of anti-ECD^{HER2} IgG2a and IgG3 response. IgG1 production is associated with a T_H2 immune response and IgG2a production with a T_H1 immune response [27]. These responses reflect the immunomodulatory effects of the fused cytokines. GM-CSF has been shown to increase antigen presentation in a variety of cells [28-33], and enhance the immune response by amplifying T-cell proliferation [34]. IL-2 can induce a similar effect through selective amplification of antigen activated T-cells expressing high levels of the high affinity IL-2 receptors [22]. IL-12 is a potent inducer of T_H1 responses [35] and in an IFN-γ dependent fashion, induces the humoral response to shift to IgG2a and IgG3 [35, 36] which is consistent with our results. In addition, the Ab-cytokine fusion proteins may influence anti-ECD^{HER2} responses by altering the uptake and processing of ECD^{HER2} by APCs as a result of APC activation by the fused cytokines, or the use of cytokine receptors on APCs for the uptake of antigen in the immune complex. Indeed, IL-2 linked to a peptide from

influenza hamagglutinin enhanced T-cell activation by affecting the antigen-presentation function of bone marrow-derived dendritic cells [37]. Although it has been shown that interaction between dansylated BSA and the anti-dansyl-(IL-2) fusion protein was necessary to enhance an anti-BSA Ab response [38], we have not yet determined whether specific interaction between Ab-cytokine fusion proteins and the soluble ECD^{HER2} is an essential requirement for eliciting tumor protection.

Transfer of sera suggested that protection against TUBO could be mediated through a humoral pathway. Characterization of the transferred immune sera suggested that the quality of the anti-ECD^{HER2} Ab response might be important for tumor protection. Even though the greatest IgG response to ECD^{HER2} was seen in mice vaccinated with ECD^{HER2} plus IgG3-(GM-CSF), sera from these mice provided little protection to naïve mice. In contrast, sera from mice immunized with ECD^{HER2} plus IgG3-(IL-12), with a less robust IgG response, provided the greatest levels of protection. The isotype profiles of the IgG response varied with the different immunization regimen with enhanced levels of both IgG2a and IgG3 anti-ECD^{HER2} seen in mice vaccinated with ECD^{HER2} plus IgG3-(IL-12). In an earlier study it was suggested that IgG2a induced by IL-12 in mice was associated with the eradiation of tumor metastasis [39]. The role of IgG3 in tumor protection is not known. It has been shown that HER2/*neu* immunization can induce Abs that stimulate the *in vitro* growth of SK-BR-3 [21, 40], however, we found that SK-BR-3 growth was inhibited when incubated with immune sera, with the strongest inhibition observed with sera containing the highest levels of anti-ECD^{HER2} IgG. Thus vaccination of patients with ECD^{HER2} together with Ab-cytokine fusion proteins may elicit anti-ECD^{HER2} Abs capable of inhibiting the growth of HER2/*neu* expressing malignancies.

The extent of splenocyte proliferation and IFN-γ secretion in the presence of soluble ECD^{HER2} *in vitro* also depended on the vaccination regimen. Splenocytes from mice vaccinated with ECD^{HER2} plus IgG3-(GM-CSF), but not from mice vaccinated with ECD^{HER2} plus IgG3-(IL-12) showed an increased *in vitro* proliferative response to soluble ECD^{HER2} with accompanying secretion of IFN-γ. Interestingly, we found an inverse correlation between the ability of immune sera to protect naïve mice and the level of cellular activation. In mice vaccinated with ECD^{HER2} plus IgG3-(GM-CSF), the robust secretion of IFN-γ by activated T-cells could enhance the cytotoxic potential of effector cells such as granulocytes, macrophages, and monocytes [22, 41-43], and upregulate the expression of Fc receptors [42-49] required in Ab-dependent cellular cytotoxicity (ADCC) [39, 50]. Fc receptor expressing cytotoxic effector cells may be recruited to tumor by anti-ECD^{HER2} Abs. Indeed, in an earlier study it was suggested that anti-rat HER2/*neu* Abs were responsible for the infiltration of PMNs seen when TUBO was implanted into rat HER2/*neu*-DNA vaccinated mice [6]. In the current studies we have not addressed the role of cytotoxic T-lymphocytes in the observed protection.

Vaccine strategies targeting HER2/*neu* might be more effective in the treatment of patients with HER2/*neu* expressing cancers than passive infusion of monoclonal anti-HER2/*neu* Abs. Passive Abs are cleared from circulation, which may lessen their therapeutic potential. Optimal circulating levels of Trastuzumab (Herceptin) were essential for a clinical response in patients [51] and an effective vaccine targeting HER2/*neu* may provide a continuous supply of anti-HER2/*neu* Abs. Immunogenicity of monoclonal antibodies [23, 52, 53] may also limit their long term use whereas antibodies produced by the endogenous immune response would not be expected to be

immunogenic. Ab-cytokine fusion proteins may also trigger a potent anti-tumor response when administered to patients with high levels of circulating soluble HER2/*neu*.

These studies have now shown that ECD^{HER2} protein vaccination using immunoenhancing Ab-cytokine fusion proteins can provide potent anti-tumor activity in animal models. While the strategy used in this study was essentially a prophylactic one, future studies will address the efficacy of this approach for treatment of established tumors. Additionally, individuals who are at "high-risk" to develop cancer may benefit from prophylactic regimens geared to suppress tumor growth. Our results suggest that although a similar extent of anti-tumor activity is seen following vaccination with the different Ab-cytokine fusion proteins, humoral and cellular immune responses may make variable contributions to protection under the different regimens. A better fundamental understanding of the requirements for a protective immune responses may lead to the design of effective vaccine regimens for use in the clinic.

Acknowledgments

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Appendix

The abbreviations used in this paper are: ECD^{HER2}, extracellular domain of HER2/*neu* antigen; ICD, intracellular domain of HER2/*neu* antigen; AP, alkaline phosphatase; ADCC, antibody dependent cellular cytotoxicity; PMN, polymorphonuclear.

Tables

Table I a. *Murine IgG1 anti-ECD^{HER2} titers* [a].

Mouse No.	PBS	ECD	IgG3	IgG3-(GM-CSF)	IgG3-(IL-2)	IgG3-(IL-12)
1	0[b]	81000	9000	243000	81000	0[b]
2	0[b]	27000	27000	243000	27000	3000
3	0[b]	27000	27000	243000	81000	9000
4	0[b]	27000	27000	243000	81000	9000
5	0[b]	81000	27000	81000	81000	9000
6	0[b]	9000	27000	81000	243000	9000
7	0[b]	0[b]	27000	81000	81000	27000
8	0[b]	9000	81000	243000	81000	3000
Average:	0	32,625	31,500	182,250	94,500	8,625

Table I b. *Murine IgG2a anti-ECD^{HER2} titers* [a].

Mouse No.	PBS	ECD	IgG3	IgG3-(GM-CSF)	IgG3-(IL-2)	IgG3-(IL-12)
1	0[b]	0[b]	0[b]	800	400	800
2	0[b]	0[b]	0[b]	100	0[b]	3200
3	0[b]	0[b]	200	400	3200	800
4	0[b]	0[b]	0[b]	100	100	6400
5	0[b]	0[b]	0[b]	400	200	6400
6	0[b]	0[b]	0[b]	0[b]	100	12800
7	0[b]	0[b]	0[b]	100	800	12800
8	0[b]	100	0[b]	200	1600	12800
Average:	0	13	25	263	800	7,000

[a] Groups of eight female BALB/c mice were injected s.c. in the right flank with either PBS, ECD^{HER2} alone, ECD^{HER2} plus IgG3, ECD^{HER2} plus IgG3-(GM-CSF), ECD^{HER2} plus IgG3-(IL-2), or ECD^{HER2} plus IgG3-(IL-12), at week 0 and again at week 5. At week 8, sera were examined for anti-ECD^{HER2} (a) IgG1 and (b) IgG2a titers by ELISA. IgG1 levels were determined using sera diluted 1:1000 and further diluted 1:3. IgG2a levels were determined using sera diluted 1:50 and further diluted 1:2. Values represent the average of duplicate dilutions of serum required to yield an absorbance $OD_{410\,nm} \geq 0.05$ after 2 hrs of incubation.
[b] Absorbance at $OD_{410\,nm} < 0.05$ at 1:1000 (a) or 1:50 (b) initial sera dilution.

Table II. *Passive transfer of immunity* [a].

	Average Tumor size (mm^3)					
Groups	Day 7	Day 10	Day 13	Day 16	Day 19	Day 21
Control	51	219	439	771	1045	1581
PBS	50	184	452	589	983	1468
ECD	93	160	451	595	932	1681
IgG3	54	316	432	699	1242	1604
IgG3-(GM-CSF)	52	140	**233**	510	897	1077
IgG3-(IL-2)	39	150	261	**381**	**605**	977
IgG3-(IL-12)	19	135	**161**	**389**	**489**	**804**

[a] Groups of 6 female BALB/c mice were injected i.v. with 175 µl of pooled immune sera. The following day, day 0, 10^6 TUBO cells were injected s.c. in the right flank. Tumor growth was examined and measured beginning on day 7 and every three days until day 21. Underlined-bold values indicate the average tumor size of mice in each group with p values ≤ 0.05 compared to the average tumor size of untreated mice.

Figure Legends

Figure 1. Tumor growth in vaccinated mice challenged with TUBO. Groups of eight female BALB/c mice were vaccinated s.c. on day –56 and again on day –21 with PBS (●), ECD^{HER2} protein alone (ECD) (□), ECD^{HER2} plus IgG3 (IgG3) (◆), ECD^{HER2} plus IgG3-(GM-CSF) (IgG3-(GM-CSF)) (◇), ECD^{HER2} plus IgG3-(IL-2) (IgG3-(IL-2)) (■) or ECD^{HER2} plus IgG3-(IL-12) (IgG3-(IL-12)) (○). On day 0, 10^6 TUBO cells were injected s.c. in the left flank. **(A)** Individual tumor size were measured starting on day 7 and every three days until day 19. **(B)** Survival curve. Mice with tumors exceeding 1.5 cm in diameter at the time of inspection were euthanized and considered to have not survived the challenge.

Figure 2. Influence of sera on the *in vitro* proliferation of SK-BR-3 cells. SK-BR-3 cells were incubated with complement inactivated pooled immune sera obtained from vaccinated mice two days prior to the challenge with TUBO cells for 6 days. Wells were pulsed with ^{3}H-thymidine 12 hrs prior to the end of the incubation. Immune sera were diluted 1:100 **(A)**, or 1:300 **(B)**. The error bars represent the range of values obtained. **(C)** Anti-ECD^{HER2} response. The anti-ECD^{HER2} IgG levels in the pooled sera were determined by ELISA. Mice treated with PBS showed undetectable levels of anti-ECD^{HER2} IgG and were used as a blank. Values represent the average intensity at $OD_{410\ nm}$ of duplicate wells at the indicated serum dilution. The error bars indicate the range of duplicate values.

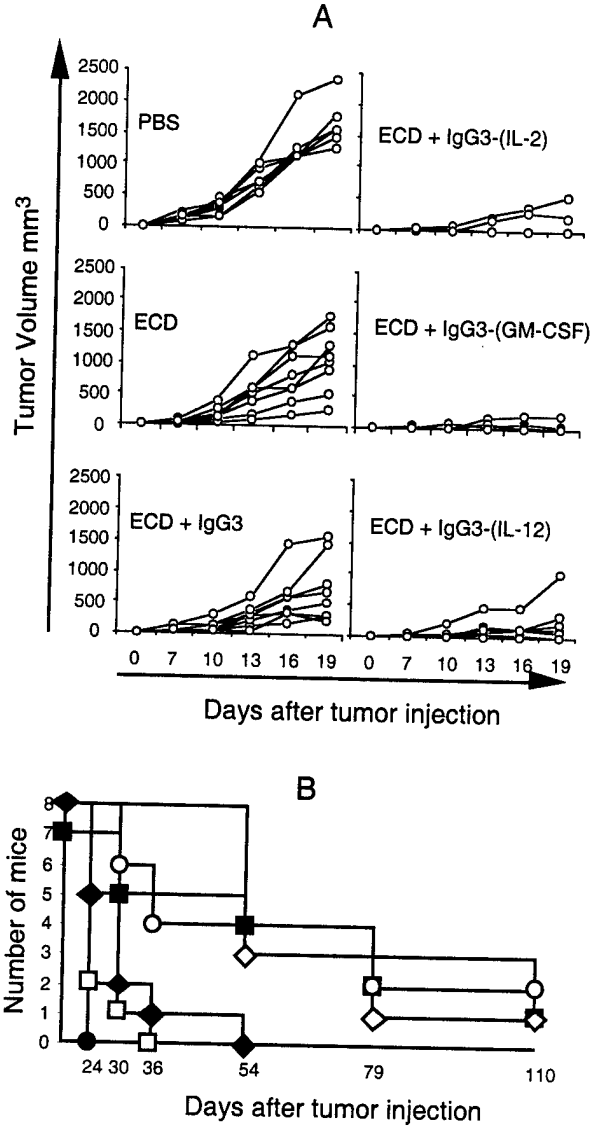
Figure 3. Characterization of anti-ECD^{HER2} IgG of transferred immune sera. Pooled sera from vaccinated mice were examined for anti-ECD^{HER2} **(A)** IgG1, **(B)** IgG2a and **(C)** IgG3 levels by ELISA. Values represent the average intensity at $OD_{410\,nm}$ of duplicate wells at 1:1000, 1:50 or 1:50 dilutions, respectively. Sera from mice injected with PBS were used as a blank. The error bars represent the range of duplicate determination.

Figure 4. *In vitro* stimulation of proliferation by ECD^{HER2} protein. Pooled splenocytes from vaccinated mice were incubated with soluble ECD^{HER2} in a 24-well tissue culture plate and pulsed with ^{3}H-thymidine 12 hrs prior to the end of the incubation periods: **(A)** 48 hrs or **(B)** 96 hrs. The stimulation index was determined as described in the Materials and Methods. The error bars represent the range of quadruplicate values.

Figure 5. *In vitro* IFN-γ production by stimulated splenocytes from vaccinated mice. Supernatants from splenocytes of vaccinated mice were harvested after **(A)** 36 hrs or **(B)** 84 hrs of incubation with soluble ECD^{HER2}, and the level of IFN-γ secretion quantified using a sandwich ELISA. A standard curve was generated in each plate and data presented as the concentration of IFN-γ (pg/ml) minus the background (PBS control) levels. The error bars represent the range of duplicate values.

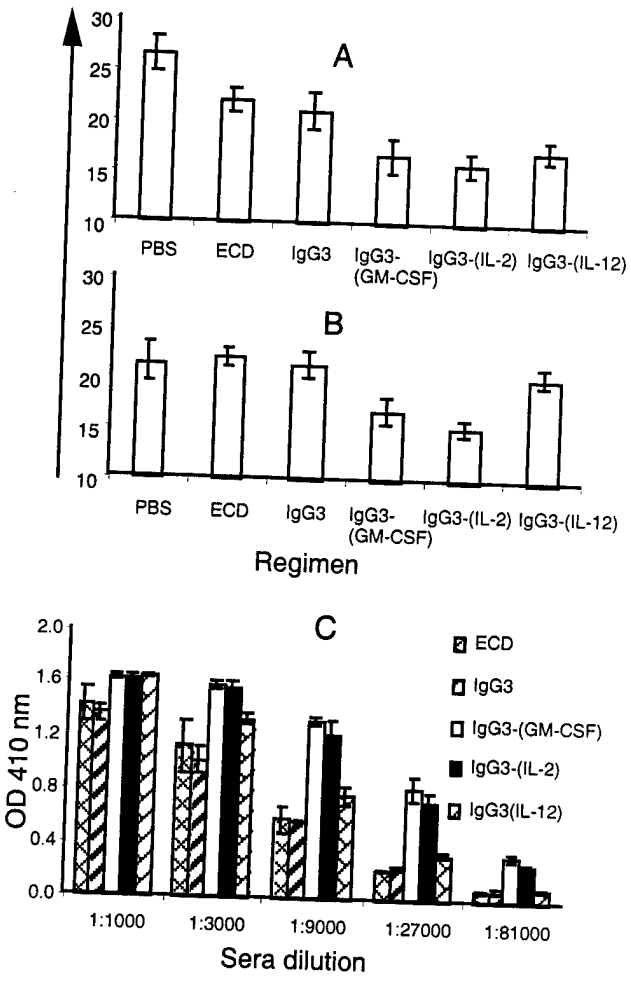
Protein vaccination with the HER2/*neu* extracellular domain plus anti-HER2/*neu* antibody-cytokine fusion proteins induces a protective anti-HER2/*neu* immune response in mice.

Dela Cruz, J.S. et al.
Figure 1.



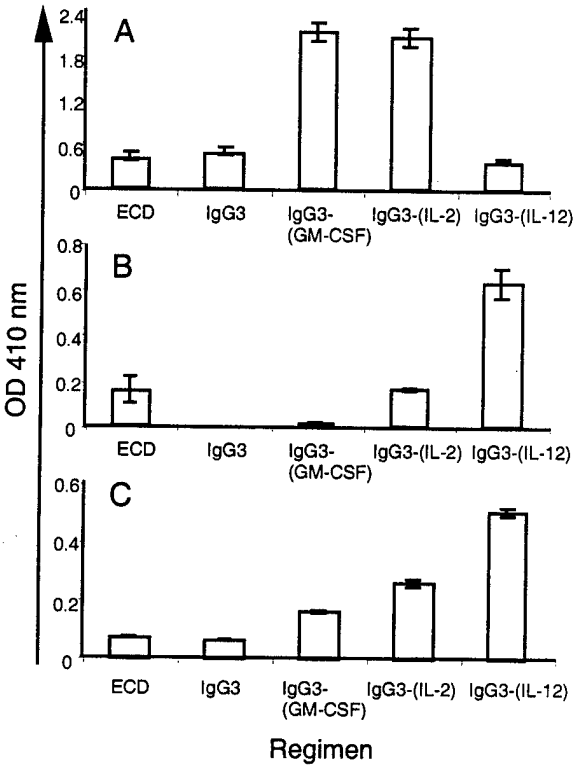
Protein vaccination with the HER2/*neu* extracellular domain plus anti-HER2/*neu* antibody-cytokine fusion proteins induces a protective anti-HER2/*neu* immune response in mice.

Dela Cruz, J.S. et al.
Figure 2.



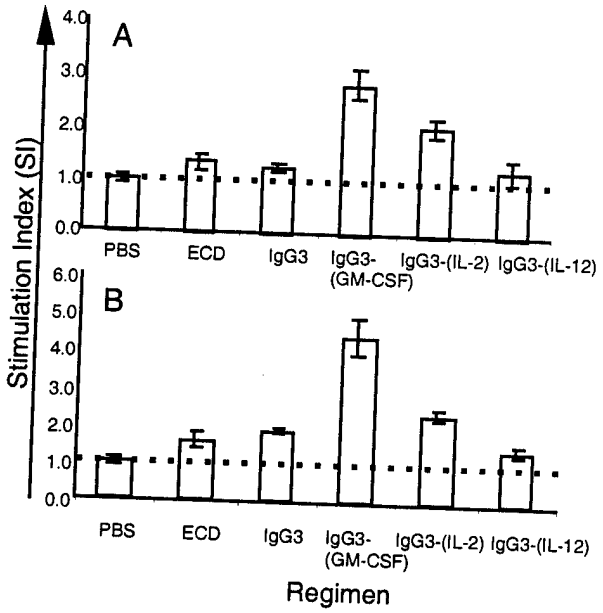
Protein vaccination with the HER2/*neu* extracellular domain plus anti-HER2/*neu* antibody-cytokine fusion proteins induces a protective anti-HER2/*neu* immune response in mice.

Dela Cruz, J.S. et al.
Figure 3.



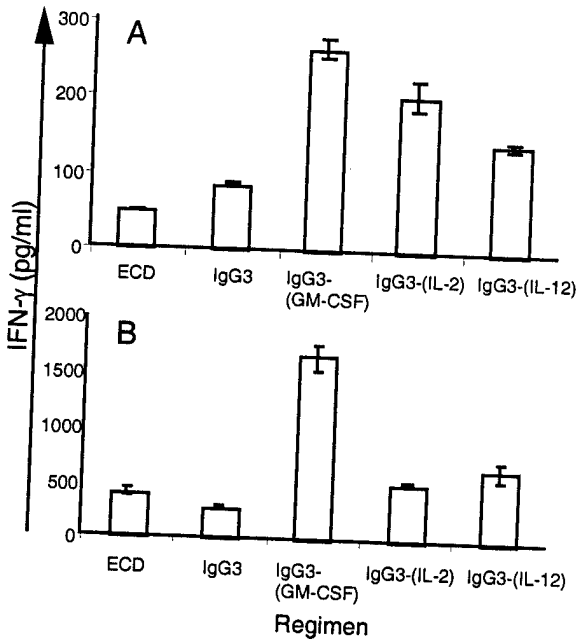
Protein vaccination with the HER2/*neu* extracellular domain plus anti-HER2/*neu* antibody-cytokine fusion proteins induces a protective anti-HER2/*neu* immune response in mice.

Dela Cruz, J.S. et al.
Figure 4.



Protein vaccination with the HER2/*neu* extracellular domain plus anti-HER2/*neu* antibody-cytokine fusion proteins induces a protective anti-HER2/*neu* immune response in mice.

Dela Cruz, J.S. et al.
Figure 5.



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Appendix 6

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Review article

Antibody–cytokine fusion proteins for the therapy of cancer

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Abstract

Advances in genetic engineering and expression systems have led to rapid progress in the development of antibodies fused to other proteins. These 'antibody fusion proteins' have novel properties and include antibodies with specificity for tumor associated antigens fused to cytokines such as interleukin-2 (IL2), granulocyte/macrophage colony-stimulating factor (GM-CSF), and interleukin-12 (IL12). The goal of this approach to cancer therapy is to concentrate the cytokine in the tumor microenvironment and in so doing directly enhance the tumoricidal effect of the antibody and/or enhance the host immune response (T-cell, B-cell or NK) against the tumor. In the past decade, multiple antibody–cytokine fusion proteins have been developed with different specificities targeting a broad variety of tumors. These novel molecules retain both antibody and cytokine associated functions. In addition, in animals bearing tumors, antibody–cytokine fusion proteins are able to target the tumor and to elicit a significant anti-tumor response that in some cases results in a complete elimination of the tumor. These results suggest that antibody–cytokine fusion proteins have potential for use in the treatment of human cancer. In the present review, we describe strategies for construction of antibody–cytokine fusion proteins and discuss the properties of several antibody–cytokine fusion proteins with IgG genetically fused to the cytokines IL2, GM-CSF or IL12.

Keywords: Antibodies; Cytokines; Immunotherapy; Antibody fusion protein; Immunocytokine; Cancer

1. Introduction

The management of residual disease is a central problem in the treatment of cancer. Despite improvements in treatment protocols, relapse remains a critical and generally fatal problem in high risk cancer patients. Chemotherapeutic strategies are necessarily limited by various toxicities and of limited efficacy. Therefore, additional modalities are needed to achieve disease containment or elimination. Systemic treatment with cytokines such as IL2, IL12 and GM-CSF can render some non-immunogenic tumors immunogenic, activating a protective immune response (Rosenberg et al., 1998; Ruef and Coleman, 1990; Tsung et al., 1997). However, when

Abbreviations: Ab, antibody; Ag, antigen; TAA, tumor-associated antigen; IL2, interleukin-2; rhIL2, recombinant human IL2; IL12, interleukin-12; rmIL12, recombinant murine IL12; GM-CSF, granulocyte/macrophage colony-stimulating factor; rmGM-CSF, recombinant murine GM-CSF; Ig, immunoglobulin; NK, natural killer; DNS, *N,N*-dimethyl-1-aminonaphthalene-5-sulfonyl chloride (dansyl); LAK, lymphokine activated killer; E/T, effector target; Id, idiotype; i.p., intraperitoneal; s.c., subcutaneous; i.v., intravenous; PBS, phosphate-buffered saline; ADCC, antibody dependent cell-mediated cytotoxicity; MAb, monoclonal antibody; CTL, cytotoxic T lymphocyte; scFv, single chain Fv; KSA, KS antigen; Ep-CAM, epithelial cell adhesion molecule; CHO, chinese hamster ovary; MHC, major histocompatability complex; NHL, non-Hodgkin's lymphoma; PBMC, peripheral blood mononuclear cells

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cytokines are given systemically there are frequently problems with severe toxic side effects that make it impossible to achieve an effective dose at the site of the tumor (Cohen, 1995; Maas et al., 1993; Ruef and Coleman, 1990; Siegel and Puri, 1991).

More effective treatment with cytokines could be achieved if methods were developed to provide effective concentrations in the tumor while limiting generalized toxicity. Direct injection into the site of the tumor has been one approach to this problem (Cortesina et al., 1988; Forni et al., 1987; Maas et al., 1989, 1991; Pizza et al., 1984; Rutten et al., 1989). However, this requires that the tumor be localized and accessible and direct injection into micrometastases is not possible. Another approach has been cytokine gene therapy whereby tumor cells are removed from the patients, transduced or transfected with the cytokine of interest, and reintroduced into the patient with the expectation that a systemic immune response will be elicited against the tumors (Dranoff and Mulligan, 1995; Hurford et al., 1995; Maass et al., 1995; Schmidt et al., 1995; Soiffer et al., 1998; Su et al., 1994; Zatloukal et al., 1995). Although the results suggest that this immunization strategy has potential application in the treatment of minimal residual disease, the ex vivo genetic modification and reintroduction of cells into patients is limited by its patient specific nature. Additionally, it is technically difficult, time consuming and expensive to expand primary autologous human tumor cells to the numbers required for vaccination (Hrouda et al., 1999; Simons et al., 1997, 1999; Soiffer et al., 1998). While in vivo gene delivery using viral vectors has been considered, the in vivo low transfer efficiency of viral vectors and their immunogenicity and safety limit their use (Hrouda et al., 1999). In addition, surface glycoproteins of many viral vectors bind to receptors prevalent on a variety of cells, such non-specific interaction directly decreases the transfection efficiency in vivo (Smith and Wu, 1999). Moreover, a significant fraction of the human population carries preexisting antibodies to viral vectors and such unfavorable immune responses decrease the half-life of vectors (Piedra et al., 1998). Thus the challenge is to develop an alternative approach for achieving effective local concentrations of cytokines.

Tumor specific Abs genetically fused to cytokines provide an alternative approach for concentrating in the region of the tumors quantities of cytokine sufficient to elicit a significant anti-tumor activity without accompanying systemic toxicity. In fact, in the past decade, we and others have developed several Ab-cytokine fusion proteins specific for different TAAs. In preclinical trials using murine model such Ab-cytokine fusion proteins have been shown to be very effective anti-cancer agents suggesting that they may be useful in the treatment of human cancer. As the number and diversity of Ab-cytokine fusion proteins has dramatically increased in the last years the present review cannot include all of them. Instead we will focus this review on a subset of Ab-cytokine fusion proteins consisting of IgG genetically fused to the cytokines IL2, GM-CSF or IL12.

2. Ab-(IL2) fusion proteins

Originally known as 'T cell growth factor', IL2 is a cytokine produced by T helper cells which stimulates T cells to proliferate and become cytotoxic (Grimm et al., 1982; Hank et al., 1990; Lotze et al., 1981; Yron et al., 1980) and NK cells to respond with increased cytotoxicity toward tumor cells (Grimm et al., 1982). These properties suggest that Ab-IL2 fusion proteins targeting cancer cells may be effective for cancer treatment. Indeed, among the Ab-cytokine fusion proteins, Ab-IL2 fusion proteins have been the best characterized and most broadly used in successful anti-tumor experiments using animal models.

Although our long term goal was to provide tumor specific therapeutics, the first IgG3-IL2 fusion protein that we produced in the laboratory was specific for the hapten DNS (Harvill and Morrison, 1995) and had human IL2 was fused after the end of the C_H3 domain of human IgG3. Human IgG3 as used since its extended hinge region is expected to provide spacing and flexibility (Dangl et al., 1988). In addition, IgG3 is the most effective of the human isotypes in complement activation (Tao and Morrison, 1989) and like IgG1 binds all three FcγRs (Roitt et al., 1989). The anti-DNS IgG3-IL2 expressed in myeloma cells was properly assembled and secreted. Anti-DNS IgG3-IL2 retains the ability

to bind Ag, activate complement and bind FcγRI (Harvill and Morrison, 1995). It resembles rhIL2 in its ability to stimulate proliferation of the IL2 dependent cell line CTLL-2 (Harvill and Morrison, 1995). Surprisingly, anti-DNS IgG3-IL2 was more effective than rhIL2 in generating LAK cell activity, eliciting more efficient killing than a 10 fold higher concentration of rhIL2 at all E/T ratios (Harvill and Morrison, 1995). This increased activity may reflect the fact that anti-DNS IgG3-IL2 exhibits approximately a 4-fold higher affinity than native IL2 for the α chain of the high affinity IL2 receptor (Table 1) (Harvill and Morrison, 1996).

Anti-DNS IgG3-IL2 has a half-life in mice of approximately 7 h (Harvill et al., 1996). Although this half-life is shorter than the half-life of IgG3, it is 17-fold longer than the half-life reported for IL2 (Donohue and Rosenberg, 1983; Talmadge et al., 1987) and is similar to that observed for polyethylene glycol-modified IL2 (PEG-IL2) which in some cases exhibits superior anti-tumor efficacy compared with IL2 (Katre et al., 1987; Mattijssen et al., 1992). Indeed this increased half-life may contribute to a better therapeutic effect (Lotze et al., 1985). We also found that, anti-DNS-IL2 was effective in potentiating the immune response to dansylated antigens (Harvill et al., 1996).

To test the effectiveness of IgG3-IL2 fusion proteins in treating the highly malignant murine B cell lymphoma 38C13 (Penichet et al., 1998), the variable regions of the anti-DNS IgG3-IL2 fusion protein were replaced with the variable regions of a murine MAb specific for the Id of the Ig expressed on the surface of 38C13. The resulting anti-Id IgG3-IL2 exhibited in vitro properties and in vivo half-life similar to anti-DNS IgG3-IL2.

Gamma camera imaging studies showed that ^{131}I-anti-Id IgG3-IL2 has the ability to target the tumor thereby concentrating the IL2 at this site (Fig. 1) (Penichet et al., 1998). A small amount of ^{131}I-anti-DNS IgG3-IL2 was localized to the upper abdomen of the animal in the region of the spleen, an organ rich in IL2 receptors 24 h after injection (Fig. 1A). In contrast, the majority of ^{131}I-anti-Id IgG3-IL2 fusion protein present at 24 h was localized to the right flank, the site of the tumor (Fig. 1B) with a little localized in the region of the spleen. At 4 days

Table 1
Rate constants[a]

Ligand	k_a (M^{-1} s^{-1})	k_d (s^{-1})	K_D (M)
rhIL-2	7.0×10^{4}	0.013	1.9×10^{-7}
IgG3-IL2	7.5×10^{4}	0.004	5.3×10^{-8}

[a] A soluble form of the α subunit of the IL2 receptor was immobilized on the surface of an IAsys optical biosensor. Association of both rhIL-2 and IgG3-IL2 at various concentrations to the a subunit was monitored over time. Following association the cuvette was washed with PBS/Tween, and dissociation followed. k_{on} values calculated using the FastFit program were plotted against the concentration with the slope of the best fit line yielding k_a. k_d was determined both from the y intercept of the graph and from direct analysis of the dissociation curves using the FastFit program. K_D was calculated as k_d/k_a.

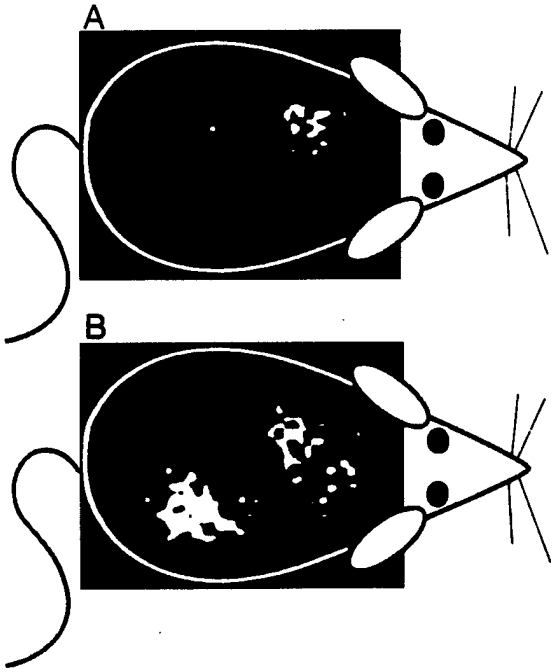

Fig. 1. Tumor localization of Ab fusion proteins. C3H/HeN mice bearing s.c. 38C13 tumors of 0.5 cm diameter on the right flank were given a single i.p. injection of 10 μCi (2 μg) ^{131}I-labeled anti-DNS IgG3-IL2 (A) or anti-Id IgG3-IL2 (B). Then, 24 h later mice were visualized with a Raytheon Spectrum 91 gamma camera. The mice were sedated and immobilized in a prone position with the pinhole collimeter positioned over the torso. A total of 50 000 count images were accumulated and analyzed using Siemens/Microdelta and Adobe software. Reprinted from Penichet et al., 1998. An IgG3-IL2 fusion protein recognizing a murine B cell lymphoma exhibits effective tumor imaging and anti-tumor activity. J. Interferon Cytokine Res., 18: 597–607.

^{131}I-anti-Id IgG3-IL2 could still be seen at the site of the tumor (data not shown). Less definitive imaging was obtained with the ^{131}I-anti-Id IgG3 which remained distributed throughout the abdomen at 24 h and exhibited only modest accumulation at the site of the tumor at 4 days (data not shown). Images taken immediately after injection showed similar uniform distribution throughout the abdomen for all three proteins (data not shown). These results suggest that tumor specific Ab-IL2 fusion proteins may also be effective imaging agents.

The superior tumor imaging of ^{131}I-anti-Id IgG3-IL2 compared to ^{131}I-anti-Id IgG3 may reflect several parameters. The more rapid clearance of the Ab-IL2 fusion protein as compared with the Ab may result in the elimination of unbound protein, thereby decreasing the background. In addition, IL2 has been shown to increase vascular permeability causing the efflux of intravascular fluids to the extravascular compartments (Damle and Doyle, 1989; Ohkubo et al., 1991). Therefore, the IL2 concentrated in the tumor area might increase the tumor uptake of the radiolabeled molecule. In fact, it has been reported that pre-treatment of nude mice bearing the Raji lymphoma with a IgG1–IL2 fusion protein (chLym-1/IL2) containing the variable region of the anti-human B-cell lymphoma MAb Lym-1 enhances the uptake of the radiolabeled mouse albumin and mouse–human chimeric IgG1 Lym-1 (chLym-1) at the tumor site. The chLym-1/IL2 is thought to alter the permeability of tumor vessels (Hu et al., 1996). Similarly, pretreatment with an Ab-IL2 fusion protein directed against DNA enhances the delivery of therapeutic molecules to solid tumors (Hornick et al., 1999). These results suggest that Ab-IL2 fusion proteins can be used to enhance the targeting of secondary anti-tumor molecules for the treatment of human malignancies.

Anti-Id IgG3-IL2 shows enhanced anti-tumor activity compared to the combination of Ab and IL2 administered together (Penichet et al., 1998). Treatment of mice bearing i.p. tumors with a single i.p. injection of 10 μg of fusion protein prevented tumor growth in 50% of the mice (Table 2, experiment 1). Rechallenge of the survivors indicated immunologic memory with varying degrees of protection consistent with the possibility of a specific T cell response. Consistent with this conclusion immunohistochemical analysis of the tumor from one long term survivor showed extensive infiltration of $CD8^+$ cells. In addition similar treatment of nude mice defective in the ability to mount a T cell immune response did not yield improved survival. In an attempt to improve the efficacy of the therapy, mice with s.c. tumors were treated five times on consecutive days with i.p. injection of 10 μg anti-Id IgG3-IL2. Indeed this approach was more effective resulting in 87% disease-free survivors (Table 2, experiment 2). Although the tumor specific fusion protein was dramatically more effective than non-tumor specific anti-DNS IgG3-IL2 showing the importance of tumor targeting, anti-DNS IgG3-IL2 was more effective than IL2 alone with two of eight treated mice disease-free survivors (25%) (Table 2, experiment 2). This result suggests that IL2 with a much longer half-life might elicit an effective anti-tumor response without direct tumor targeting.

In contrast to what was observed following a single dose of 10 μg of fusion protein, rechallenge of the mice surviving after five doses provided little evidence for immunologic memory (Penichet et al., 1998) suggesting that specific cytotoxic T cells did not play a significant role in providing protection.

Table 2
Results of in vivo therapy experiments

Group	Treatment	Disease free survivors[a]	
		Experiment 1[b]	Experiment 2[c]
1	PBS	0/6 (0%)	0/8 (0%)
2	Anti-Id IgG3	1/6 (16.7%)	2/8 (25%)
3	IL-2	0/6 (0%)	0/8 (0%)
4	Anti-Id IgG3+IL-2	0/6 (0%)	4/8 (50%)
5	Anti-Id IgG3-IL2	3/6 (50%)	7/8 (87.5%)
6	Anti-DNS IgG3-IL2	Not done	2/8 (25%)

[a] Animals surviving 60 days without evidence of tumor were considered to be tumor free.

[b] Groups of six C3H/HeN mice were injected i.p. with 1000 38C13 cells. The following day each group received single i.p. injections of: PBS, 10 μg of anti-Id IgG3, 30 000 IU of rhIL-2, both 10 μg anti-Id IgG3 and 30 000 IU of rhIL-2, or 10 μg of anti-Id IgG3-IL2.

[c] Groups of eight C3H/HeN mice were injected s.c. with 1000 38C13 cells. The following day each group received the first of five daily i.p. injections. Treatments included PBS, 10 μg of anti-Id IgG3, 30 000 IU of rhIL-2, both 10 μg anti-Id IgG3 and 30 000 IU of rhIL-2, 10 μg of anti-Id IgG3-IL2 or 10 μg of an irrelevant human IgG3-IL2 fusion protein without binding specificity for 38C13 (anti-DNS IgG3-IL2).

These results are consistent with the previous reports that low doses of IL2 activate T cells while higher doses lead to NK activation (Maas et al., 1993; Talmadge et al., 1987). The dose dependence of the type of response elicited by IL2 may explain the different results obtained upon rechallenge of the survivors from the first and second trial. In fact using IL2 expressing transfected cells as cancer vaccines, it has been observed that high levels of IL2 production are associated with a failure to generate tumor specific CTLs (Schmidt et al., 1995).

Studies from other laboratories have shown that a mouse–human chimeric anti-Id IgG1–mouse IL2 fusion protein (chS5A8-IL2) was more effective in the in vivo eradication of the 38C13 tumor than the combination of the anti-Id Ab and IL2 or irrelevant Ab-IL2 fusion protein (Liu et al., 1998). Interestingly an anti-Id scFv–IL2 fusion protein (scFvS5A8-IL2) containing the variable regions of the chS5A8-IL2 failed to confer protection suggesting the relevance of the Fc effector functions such as ADCC in anti-tumor activity against 38C13 (Liu et al., 1998).

Remarkable success in preclinical trials using syngeneic murine models bearing melanoma, neuroblastoma, and colorectal carcinoma and treated with specific mouse–human chimeric or humanized Ab-human IL2 fusion proteins has been reported by both the Reisfeld and Gillies groups (Becker et al., 1996a,b,c; Lode et al., 1998; Xiang et al., 1997, 1999). To evaluate the in vivo anti-tumor activity of the mouse–human chimeric anti-GD_2 IgG1-IL2 fusion protein ch14.18-IL2, B16 melanoma cells were transduced with human genes encoding the two enzymes in the last stages of diasialoganglioside GD_2 biosynthesis (Becker et al., 1996a). The resulting cell line expressed GD_2, a ganglioside present on the surface of human melanoma but not murine melanoma. Treatment of mice bearing pulmonary and hepatic metastases as well as subcutaneous GD_2 expressing B16 melanoma with ch14.18-IL2 resulted in specific and strong anti-tumor activity. This anti-tumor activity was highly significant compared to the controls such as Ab (ch14.18) and IL2 or irrelevant Ab-IL2 fusion proteins and resulted in the complete eradication of the tumor in a vast number of animals (Becker et al., 1996b,c). It was also found that the immune response induced in tumor-bearing animals receiving treatment with ch14.18-IL2 was $CD8^+$ T-cell dependent and was followed by long-lived transferable protective immunity (Becker et al., 1996a,b,c). The encouraging preclinical results obtained with ch14.18-IL2, have led to Phase I clinical trial using a humanized version of ch14.18-IL2 (Hu14.18-IL2) in patients with GD_2 melanomas (Ostendorf et al., 2000).

GD_2 is also extensively expressed on human but not murine neuroblastoma. A murine neuroblastoma expressing GD_2, NXS2, was established by hybridization of dorsal root ganglional cells of C57BL/6J mice with C1300 mouse neuroblastoma cells (Lode et al., 1998). Using NXS2, ch14.18-IL2 was found to induce a cell-mediated anti-tumor response that effectively eradicated established bone marrow and liver metastases in a syngeneic model. However, the mechanism involved was exclusively NK dependent (Lode et al., 1998) illustrating the fact that different mechanisms of anti-tumor activity may result when different murine tumors expressing the same Ag are treated with the same Ab–cytokine fusion protein.

To evaluate the treatment of colorectal carcinoma using a human TAA as target and immunocompetent mice, the murine colon carcinoma cell line CT26 was transduced with the gene encoding human KSA, an Ep-CAM expressed in a human colon carcinoma and other human malignancies derived from epithelial tissue (Xiang et al., 1997). Like the results obtained with ch14.18-IL2 against melanoma, treatment of mice bearing CT26-KSA hepatic and pulmonary metastases with the humanized anti-KSA Ab-IL2 fusion protein huKS1/4-IL2 directs IL2 to the tumor microenvironment in concentrations that are sufficient to elicit a specific $CD8^+$ T-cell-mediated eradication of established metastases and long-lived T cell memory (Xiang et al., 1997, 1999).

3. Ab-(GM-CSF) fusion proteins

GM-CSF is a cytokine associated with the growth and differentiation of hematopoietic cells. It is also a potent immunostimulator with pleiotropic effects, including the augmentation of Ag presentation in a variety of cells (Blanchard and Djeu, 1991; Fischer et al., 1988; Heufler et al., 1988; Morrissey et al., 1987; Smith et al., 1990; Steis et al., 1990), increased expression of MHC class II on monocytes

and adhesion molecules on granulocytes and monocytes (Arnaout et al., 1986; Grabstein et al., 1986; Young et al., 1990), and amplification of T-cell proliferation (Santoli et al., 1988). All of these features suggest that localization of GM-CSF at the site of tumor by Ab-(GM-CSF) fusion proteins that recognize TAA may lead to an enhanced tumor specific immune response.

To expand the clinical potential of a promising mouse–human chimeric IgG1 (chCLL-1) directed against a human MHC class II expressed in a high percentage of human B-cell NHL, chronic lymphocytic leukemia, and multiple myeloma cell lines it was joined to human GM-CSF (chCLL-1/GM-CSF) (Hornick et al., 1997). chCLL-1/GM-CSF not only retained the Ab and GM-CSF bioactivity, but also showed enhanced ADCC activity compared to Ab (chCLL-1) using human mononuclear cells (Hornick et al., 1997). In addition, biodistribution and imaging studies in nude mice bearing the human myeloma cell line ARH-77 indicated that the fusion proteins specifically targets the tumors (Hornick et al., 1997) suggesting that this Ab fusion protein may be useful for the treatment of B-cell malignancies and other related cancers. Unfortunately, an animal tumor expressing the Ag recognized by chCLL-1 is not available (Hornick et al., 1997) and human GM-CSF is not active in mice (Ruef and Coleman, 1990) making it difficult to evaluate the anti-tumor activity of chCLL-1/GM-CSF in vivo.

To be able to investigate the in vivo efficacy in a variety of syngeneic tumor models, murine GM-CSF was genetically fused to the carboxyl terminus of a chimeric rat anti-mouse transferrin receptor Ab, ch17217 (Dreier et al., 1998). The ch17217-(GM-CSF) fusion protein retained both Ab and GM-CSF related activities and most importantly demonstrated effective anti-tumor activity by suppressing the growth of hepatic metastases of mouse neuroblastoma NXS2 cells and pulmonary metastases of mouse colon carcinoma CT26 cells in syngeneic A/J and BALB/c mice, respectively. However, it should be noted that the controls of Ab alone (ch17217) or rmGM-CSF were not included in these studies making it impossible to distinguish the in vivo role of the Ab-(GM-CSF) fusion protein from that of the Ab and/or GM-CSF (Dreier et al., 1998). Due to the universal expression of the transferrin receptor on mouse tumor cell lines, constructs like ch17217-(GM-CSF) should prove effective against a wide variety of murine tumors. However, the transferrin receptor is also expressed on all normal cells except mature red blood cells (Dowlati et al., 1997) and antigens whose expression is restricted to tumors may be a more appropriate target for Ab–cytokine fusion proteins. Further studies are required to evaluate the effectiveness and mechanism of action of Ab–(GM-CSF) fusion proteins in this and other tumor models.

4. Ab–(IL12) fusion proteins

IL12, a cytokine normally released by professional Ag-presenting cells, promotes cell-mediated immunity (Trinchieri, 1995) by inducing naive $CD4^+$T cells to differentiate into Th1 cells (Gracie and Bradley, 1996; Hsieh et al., 1993). In addition, IL12 has the ability to enhance the cytotoxicity of NK and $CD8^+$ T cells (Farrar and Schreiber, 1993; Gately et al., 1994). Moreover, the IFN-γ produced by IL12 stimulated T and NK cells can retard tumor growth by inhibiting tumor angiogenesis (Voest et al., 1995) and enhancing immune recognition of tumor cells through up-regulated MHC expression (Wong et al., 1984). However, in contrast to cytokines like IL2 and GM-CSF, IL12 is a disulfide-linked heterodimer of two subunits p35 and p40. Bioactive IL12 requires the expression of two separate genes, p40 and p35, and correct heterodimer assembly making the construction of an Ab–IL12 fusion protein more complicated (Gubler et al., 1991).

To address this issue, Gillies et al. (1998) constructed an Ab–IL12 fusion protein in which the p35 subunit was fused to the carboxy terminus of an Ab; the p40 subunit was expressed as a separate polypeptide which must then assemble with the p35 subunit. Although this Ab–IL12 fusion protein was functional, the IL12 bioactivity was significantly lower than rmIL12 (Gillies et al., 1998).

As an alternative approach, we fused single-chain murine IL12 to anti-HER2/*neu* IgG3 (mscIL12.her2.IgG3) (Peng et al., 1999). The use of single-chain IL12 may confer greater stability to the fusion protein and ensures equimolar concentrations of the two IL12 subunits facilitating large scale

production of the Ab–IL12 fusion protein. The cDNA coding for the murine IL12 fusion protein IL12.p40.L.Dp35 with the two subunits joined with a $(Gly_4,Ser)_3$ linker was used (Lieschke et al., 1997). This protein showed a specific activity comparable to that of rmIL12 while a related construct with a different subunit order (IL12.p35.L.Dp40) showed very low activity (Lieschke et al., 1997). In an ovalbumin–IL12 fusion protein in which the p40 subunit was fused to ovalbumin, a 50-fold lower IL12 activity was observed (Kim et al., 1997) suggesting that constraint of the p40 subunit in a fusion protein may disrupt the interaction between IL12 and the IL12 receptor. Although the p40 subunit was not fused to the antibody in the Gillies et al. (1998) Ab–IL12 fusion protein, the direct fusion of the p35 subunit to the carboxy terminus of the Ab without any type of flexible linker may make the p40 subunit somewhat less accessible for receptor binding possibly explaining the 2-fold lower IL12 activity they observed. To further insure accessibility, the murine single chain IL12 was fused to the amino terminus of heavy chain of anti-HER2/*neu*-IgG3 (Peng et al., 1999). At this position proteins which require N-terminal processing or folding for activity such as NGF (McGrath et al., 1997) and human B7-1 (Challita-Eid et al., 1998) remain active. We found that mscIL12.her2.IgG3 was assembled and correctly secreted and that the presence of IL12 at the amino terminus did not interfere with the Ab's ability to bind HER2/*neu*. In addition, mscIL12.her2.IgG3 binds the IL12 receptor and resembles recombinant IL12 in its ability to stimulate proliferation of PBMC (Peng et al., 1999).

Although our long-term goal was the production of Ab–IL12 fusion proteins for therapeutic use in humans, murine IL12 was used for these initial studies because it has activity on both human and murine cells, while human IL12 has activity only on human cells. The use of murine IL12 makes it possible not only to carry out assays using human PBMC to test biologic activity, but also to perform in vivo studies to examine the effects against human HER2/*neu*-expressing murine tumors using immune competent mice. The in vivo anti-tumor activity of mscIL12.her2.IgG3 was evaluated using murine colon carcinoma cell line CT26 transduced with the cDNA encoding human HER2/*neu* (Penichet et al., 1999) which grows in immunocompetent mice while maintaining the expression of human HER2/*neu* (Penichet et al., 1999). Fig. 2 shows that mscIL12.her2.IgG3 has significant anti-tumor activity in immune competent BALB/c mice under conditions in which the anti-HER2/*neu* Ab (her2.IgG3) failed to confer protection. We observed better anti-tumor activity when treatment was started after the tumors were established (mean diameter of 8–9 mm) than when treatment was started the day after inoculation with tumor cells. This lends support to previous studies by others (Tsung et al., 1997; Zitvogel et al., 1995; Zou et al., 1995) in which better anti-tumor activity of IL12 was observed when tumors were established. They proposed that this may be because effector cells are first recruited to the tumor site, and are then activated by IL12. Further work is being carried out using CT26-HER2/*neu* and other murine tumors expressing human HER2/*neu* to determine the mechanism of action of mscIL12.her2.IgG3.

5. Conclusions

In the last decade many studies have shown that the genetic fusion of anti-cancer Ab with cytokines results in novel proteins which retain both Ab and cytokine functions and show superior anti-cancer activity compared with equivalent amount of free Ab and cytokines or non tumor specific Ab cytokine fusion proteins. These findings support the hypothesis that Ab–cytokine fusion proteins can specifically target the cytokine to the tumor microenvironment and in so doing stimulate the immune effector cells. In some cases the stimulus is sufficient to achieve complete eradication of the tumor suggesting that these unique molecules will be effective in the treatment of human cancer. However, many Ab–cytokine fusion proteins such as Ab–(GM-CSF) and Ab–IL12 fusion protein are still in a very early phase of development and additional preclinical studies are required for a better understanding of how these novel molecules work. Of special interest will be the potential synergistic effects between different Ab–cytokine fusion proteins or between Ab–cytokine fusion proteins and other anti-cancer strategies such as chemotherapy, gene therapy and tumor vaccines.

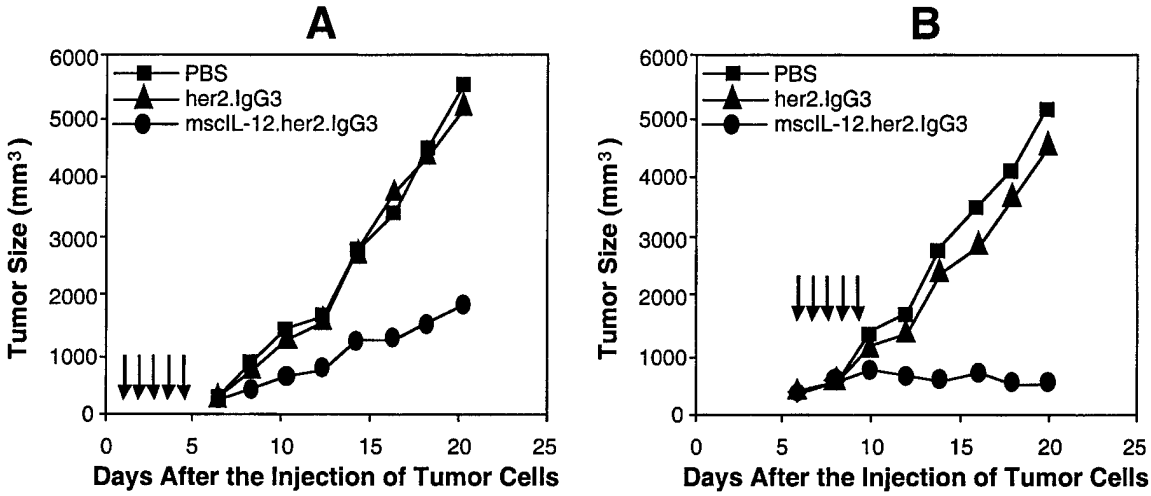

Fig. 2. In vivo anti-tumor activity. BALB/c mice were injected with 1×10^6 CT26-HER2/*neu* cells s.c. on day 0. Beginning on day 1 (A) or day 6 (B), groups of ten mice were treated i.v. with either mscIL-12.her2.IgG3 (at a concentration equivalent to 1 μg IL-12/day), her2.IgG3 (at a concentration equivalent to the Ab concentration of mscIL-12.her2.IgG3 administered/day), or PBS for 5 days. Tumor growth was measured with a caliper beginning on day 6 and tumor volume calculated. The average tumor volumes of the ten mice used in each treatment group were plotted against time. Graphs adapted from Peng et al., 1999. A single chain IL-12 IgG3 antibody fusion protein retains antibody specificity and IL-2 bioactivity and demonstrates anti-tumor activity. J. Immunol., 163: 250–258. Copyright 1999, The American Association of Immunologists.

Obviously, the ultimate use of Ab–cytokine fusion proteins for the treatment of human cancer will depend on the results obtained when these proteins are evaluated in Phase I and II clinical trials.

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Appendix 7

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Review

Myeloma expression systems

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Abstract

Myeloma expression systems have been utilized successfully for the production of various recombinant proteins. In particular, myeloma cell lines have been exploited to express a variety of different antibodies for diagnostic applications as well as in the treatment of various human diseases. The use of myeloma cells for antibody production is advantageous because they are professional immunoglobulin-secreting cells and are able to make proper post-translational modifications. Proper glycosylation has been shown to be important for antibody function. Advances in genetic engineering and molecular biology techniques have made it possible to isolate murine and human variable regions of almost any desired specificity. Antibodies and antibody variants produced in myeloma cells have been extremely helpful in elucidating the amino acid residues and structural motifs that contribute to antibody function. Because of their domain nature, immunoglobulin genes can be easily manipulated to produce chimeric or humanized antibodies. These antibodies are less immunogenic in humans and also retain their specificity for antigen and biologic properties. In addition, novel proteins in which antibodies are fused to non-immunoglobulin sequences as well as secretory IgA have been produced in myeloma cells.

Keywords: Myeloma cells; Monoclonal antibodies; Antibody engineering; Protein expression

Abbreviations: Ab, antibody; ADCC, antibody-dependent cell-mediated cytotoxicity; ASGR, asialoglycoprotein-binding receptor; Av, avidin; BBB, blood–brain barrier; CDC, complement-dependent cytotoxicity; CHO, Chinese hamster ovary cells; CMV, cytomegalovirus; C region, constant region; dhfr, dihydrofolate reductase; FcR, Fc receptor; GlcNAc, *N*-acetylglucosamine; GM-CSF, granulocyte-macrophage colony-stimulating factor; gs, glutamine synthetase; H chain, heavy chain; HPRT, hypoxanthine-guanine phosphoribosyl transferase; Id, idiotype; ID, injected dose; Ig, immunoglobulin; IGF, insulin-like growth factor; IL, interleukin; L chain, light chain; MAbs, monoclonal antibodies; MBP, mannose binding protein; NeuGc, *N*-glycolylneuramic acid; NeuAc, *N*-acetylneuraminic acid; pIgA, polymeric IgA; pIgR, poly-immunoglobulin receptor; PCR, polymerase chain reaction; PNA, peptide-nucleic acid; SC, secretory component; sIgA, secretory IgA; TAA, tumor associated antigen; Tf, transferrin; TfR, transferrin receptor; V region, variable region.

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1. Introduction

The ability to manipulate genes and express recombinant proteins have led to tremendous advances in eukaryotic expression systems. Many mammalian cell lines have been used to express recombinant proteins via transfection of plasmids or infection of recombinant DNA or RNA viruses. In particular, myeloma expression systems have been used successfully to generate monoclonal antibodies (MAbs) that are used in a variety of diagnostic, imaging and therapeutic applications. Although proteins such as the human cytokine Leukemia Inhibitory Factor (Geisse et al., 1996), soluble intercellular adhesion molecule (Werner et al., 1998) and murine CD8α-CD40 fusion protein (Lane et al., 1993) have been produced in myeloma cells, this article will focus primarily on the expression of recombinant antibodies in myeloma expression systems.

Immunoglobulins (Ig) play a critical role in the mammalian humoral immune system. Once bound, antibodies recruit effector cells and molecules to eliminate antigen. The antibody (Ab) molecule consists of two identical light (L) chains and two identical heavy (H) chains held together by disulfide bonds (Fig. 1). The antigen binding region called the variable (V) region is present at the N-terminus and varies extensively between Ab molecules, allowing them to recognize virtually any structure. The C-terminal half of the H chain (Fc) determines the distinct functional properties such as half-life and effector functions including activation of the complement cascade, binding to Fc receptors (FcR), and recruitment and activation of macrophages.

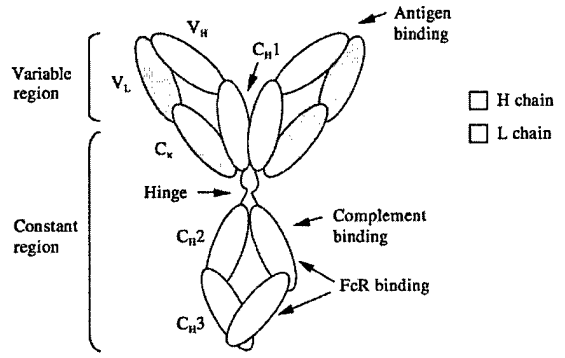

Fig. 1. Schematic diagram of a prototypic antibody molecule (IgG). Variable regions of the H (V_H) and L (V_L) chain bind antigen. The constant regions of the H (C_H1, C_H2, and C_H3) and L chain (C_κ) are also shown. The hinge region joins the Fc portion of the Ab to the Fab portion. The Fc region contains binding sites for FcRs and for complement activation.

Antibodies have proven to be valuable reagents because they can bind to a variety of ligands with exquisite specificity. In addition, the domain structure of Abs makes them amenable to protein engineering in which functional domains carrying antigen binding activities (Fabs) or effector functions (Fc) can be exchanged between Abs. The use of mutants and domain exchanged proteins has helped to elucidate the structural features on Abs that are responsible for their characteristics and contribute to effector functions. Recombinant DNA and gene expression techniques have been used to produce Abs with the desired characteristics and a variety of modifications have been made successfully to produce novel molecules such as chimeric, "humanized" and catalytic Abs, as well as Ab fusion proteins, polymeric Abs and Ab fragments. In addition, it has been possible to produce secretory IgA in myeloma cells.

2. Expression systems

A variety of eukaryotic expression systems have been used to produce recombinant proteins. The use of eukaryotic cell lines to produce proteins is advantageous because they have the ability to carry out normal post-translational modifications such as intra- and inter-chain disulfide bond formation, signal peptide cleavage, and addition of O- and N-linked carbohydrates. However, Igs produced in insect cells only contain N-linked carbohydrates with mannose as the terminal sugars. Plant cells attach different terminal sugars than do mammalian cells. These differences in glycosylation may influence in vivo biologic properties such as biodistribution, half-life, antigenicity and effector functions. Though the ability to produce large quantities of proteins in insect cells and in plants offers an economical alternative to producing therapeutic and diagnostic reagents, careful characterization of their in vivo biologic properties is essential. Alternatively, mammalian cells are well suited for recombinant protein expression because they can be correctly processed.

Myeloma cell lines have been used to produce genetically engineered Abs (Banerji et al., 1983; Gillies et al., 1983; Neuberger, 1983; Oi et al., 1983). Since they are "professional" secretory cells, myelomas are ideal for the generation of transfectomas that produce recombinant Abs. They are easy to transfect, grow naturally in suspension, allowing for large-scale production, and can be adapted to serum-free conditions. Frequently used cell lines are P3X63Ag8.653, Sp2/0-Ag14 and NSO/1. All three murine cell lines have lost the ability to produce endogenous H and L chains and are derived from the parent myeloma MOPC 21. Mineral oil injection of BALB/c mice produced MOPC 21 myeloma cells, which were then established as the P3K cell line. P3K cells were selected for those lacking hypoxanthine-guanine phosphoribosyl transferase (HPRT), resulting in the P3X63Ag8 cell line. Successive cycles of cell sorting followed by several series of cloning were performed on P3X63Ag8 cells to select for a stable cell line that was negative for intracellular H and L chain expression. The resulting cell line was P3X63Ag8.653 (Kearney et al., 1979). P3X63Ag8 cells were also fused to BALB/c spleen cells with anti-sheep red blood cell activity. The cells from the fusion were subcloned and the Sp2/0-Ag14 myeloma cell line was established (Shulman et al., 1978). NSO/1 cells were derived from NSI/1-Ag4-1 cells that were selected as nonsecreting, HPRT$^-$ P3K cells (Galfrè and Milstein, 1981).

Although the myeloma cell lines described above have similar properties and growth characteristics, they may differ in their ability to express particular Ig genes. In the case of a chimeric Ab against the *Escherichia coli* F41 antigen, transfectomas expressing the L chain were established for the Sp2/O and NSO/1 cells but not for P3X63Ag8.653 even after multiple transfections. In addition, after transfection of the H chain into the L chain producers, the NSO/1 derived transfectomas were able to produce 5- to 10-fold more intact Ab than Sp2/0 (EMY and SLM, unpublished results).

The levels of Ab production in transfectomas have generally been lower than myelomas and hybridomas, which can secrete up to 200 μg of Ab/ml of culture supernatant. Most transfectomas secrete 1–30 μg/ml, similar to the levels produced by human hybridomas and low producing murine hybridomas (Sahagan et al., 1986; Sun et al., 1987). We have observed that the amount of L chain produced by a transfectant appears to limit Ab production. As a result, we find that an efficient way of generating transfectomas producing large amounts of Ab is to first transfect in the L chain and isolate cell clones that produce the most L chain. Subsequently, the H chain expression vector is transfected. Strategies such as gene amplification and manipulation of nutrient feed composition and environmental conditions have been used to increase production levels. The use of the amplifiable marker *glutamine synthetase* (*gs*; Bebbington et al., 1992) in NS0/1 cells resulted in the production of recombinant Abs at rates of 20–50 pg/cell/day (Robinson et al., 1994). Ab production was also increased from 20 to 80 pg/cell/day in Sp2/0 cells transfected with the amplifiable marker *dihydrofolate reductase* (*dhfr*; Robinson and Memmert, 1991).

Antibodies produced in nonlymphoid cell lines such as Chines hamster ovary (CHO), HeLa, C6, and PC12 are also properly assembled and glycosylated (Cattaneo and Neuberger, 1987). To increase levels of Ab production in nonlymphoid cell lines, Ig genes have been coamplified with a linked marker such as *gs*, *dhfr* and *adenosine deaminase* (Wood et al., 1990; Page and Sydenham, 1991; Brown et al., 1992). However, the level of production in *gs*-amplified CHO cells was lower than in *gs*-amplified NSO/1 cells in large scale cultures (Brown et al., 1992). High levels of Ab (up to 100 μg/10^6 cells/24 h) were secreted in *dhfr*-amplified CHO cells and the Abs were shown to retain their ability to mediate complement-dependent cytotoxicity (CDC) and Ab-dependent cell-mediated cytotoxicity (ADCC; Page and Sydenham, 1991; Crowe et al., 1992). An added advantage of expressing Abs in CHO cells is that they are easily scaled up and can be adapted to grow under serum-free conditions.

3. Vectors for immunoglobulin expression

In initial studies, the H chain genes were expressed from the pSV2ΔH*gpt* expression vector that is derived from the pSV2 vectors developed by Mulligan and Berg (1981). The pSV2 vectors contain the β-lactamase gene and pBR322 origin of replication for selection in bacteria. A second feature of these vectors is a dominant marker *Ecogpt*, which encodes xanthine-

guanine phosphoribosyltransferase, for selection in a broad range of eukaryotic cells. The L chain vector pSV184ΔH*neo* contains the pACYC184 origin of replication, the chloramphenicol-resistance gene and the *neo* gene. The *neo* gene from a bacterial transposon encodes the enzyme aminoglycoside 3′-phosphotransferase type II. Cells expressing this enzyme can grow in the presence of G418, an antibiotic that inhibits protein synthesis (Southern and Berg, 1982). Selection for the presence of the two exogenous genes is possible since the *gpt* and *neo* genes select through two different biochemical pathways. Additional selectable markers include the *trpB* and *hisD* genes, which allow cells to grow in medium lacking the essential amino acids tryptophan and histidine, respectively (Hartman and Mulligan, 1988). *trpB* selection has been difficult to use because tryptophan released from dying cells is scavenged by the survivors (unpublished observation). *hisD* selection has been used effectively and allows the survival of cells in otherwise toxic concentrations of histidinol. Other dominant selectable markers include the *hygro* and *dhfr* genes (Wigler et al., 1980; Gritz and Davies, 1983). Exogenous genes cloned adjacent to the mutant *dhfr* gene can be amplified by selection in increasing concentrations of methotrexate.

Expression vectors must contain the appropriate transcriptional control elements such as an enhancer, a promoter, and poly(A) addition site in order to be expressed in myeloma cells. The murine H or L chain promoter and the intronic H chain and κ enhancers have been used successfully for expression of Abs in myeloma cell lines. Alternatively, strong heterologous promoters such as the human cytomegalovirus (CMV) promoter and the polyoma late promoter have been used to produce Abs in myeloma cells (Deans et al., 1984; Foecking and Hofstetter, 1986). These viral controlling elements have led to high Ab yields in myelomas and are versatile in that they can function in a variety of cell types such as CHO cells.

It is advantageous to design Ig expression vectors as cassettes to facilitate manipulation of the Ab genes (Fig. 2). Ig variable (V) and constant (C) region genes have been obtained by genomic or cDNA cloning. Somatic rearrangement of both the H and L chain variable region genes is required to produce a functional Ig molecule. For the H chain three genomic sequences, V, D, and J, must be assembled while the L chain requires the assembly of V and J segments. The expressed V region can be distinguished from the hundreds of nonexpressed Vs because only the assembled V region has an associated J region. J region probes can be used to identify the expressed V region from λ phage libraries without any prior knowledge about its sequence. One advantage of this approach is that the V

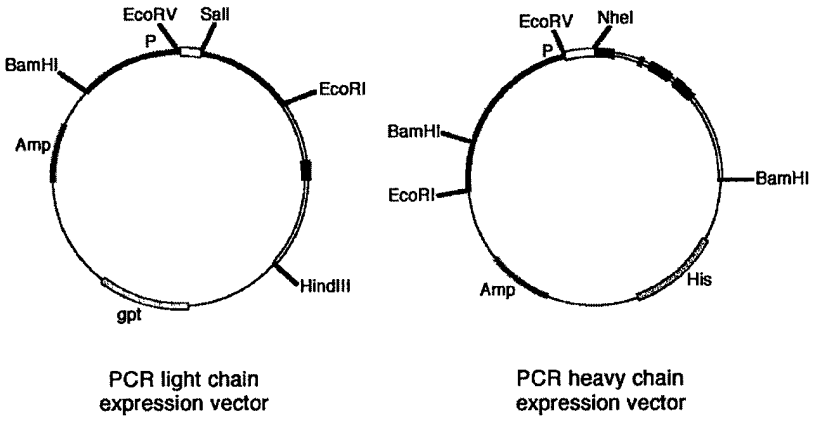

Fig. 2. Diagrams of PCR L and H chain expression vectors. The expression vectors contain unique restriction enzyme sites (*Eco*RV and *Sal*I for the L chain vector and *Eco*RV and *Nhe*I for the H chain vector) for cloning of V regions generated by PCR. The white boxes represent the murine V region exons and the black boxes the human C region exons for Cκ and Cγ. The black thick lines designate sequences from the murine genomic Ig gene and the white thick lines from the human genomic Ig gene. "P" represents the promoter. Amp is the β-lactamase gene for prokaryotic selection and gpt and His are eukaryotic selection markers.

region is obtained with its own promoter. Alternatively, J region probes can be used to identify bacteria that have been transformed with plasmids containing cDNA from Ab producing cells. However, the V regions isolated using this approach require modification before cloning and expression.

Recently, polymerase chain reaction (PCR) has been used successfully for rapid cloning and modification of V regions from Abs of many different specificities (Gillies et al., 1989; Orlandi et al., 1989; Coloma et al., 1992). Although designing primers for the 3′ end of the V region is straightforward since there are only a few C regions, designing primers for the 5′ end has been more challenging. Degenerate primers annealing to sequences in the framework region have been used; however, they introduce amino acid substitutions that may alter Ab affinity. A better approach is to use a set of redundant primers that anneal to the relatively conserved leader sequences. No mutations are introduced using this approach because the leader sequence is removed from the mature Ab molecule (Coloma et al., 1991). Primers have been designed which effectively prime both the murine and human leader sequences (Larrick et al., 1989; Gavilondo-Cowley et al., 1990; Campbell et al., 1992; Coloma et al., 1992).

Vectors that allow for the expression of V_L and V_H cloned by PCR with human C regions from genomic cloning have been described (Fig. 2; Coloma et al., 1992). Both vectors contain a murine V_H promoter with a 3′ cloning site (EcoRV). In the H chain vector, the cloning site is provided at the 5′ end of the C_H1 domain so that the V_H is cloned directly adjacent to C_H1. In the L chain vector, the restriction site is provided 3′ to a splice junction so that the V_L is amplified with an attached splice junction. These two different approaches were taken because when the V_L used in the initial studies was fused directly to the κ constant region, the gene was not expressed.

Expression vectors containing cDNA encoding the C regions of human and murine Ig H and L chain genes have recently been described (McLean et al., 2000). The expression of the Ig genes are under the control of the CMV promoter and therefore can be expressed in lymphoid and nonlymphoid cells. The vectors also contain the intronic H chain enhancer for more uniform expression in B cells. In these vectors, the V_H and V_L are directly adjacent to the C_H1 and C_κ constant regions, respectively, and contain cloning sites for V regions generated by PCR. The introduction of restriction sites for cloning resulted in one amino acid substitutions at the 5′ end of the human $C\gamma$, $C\mu$, $C\alpha1$, and C_κ vectors.

Expression vectors containing both the H and L chain genes on one plasmid have also been described (Norderhaug et al., 1997; Preston et al., 1998). This approach is advantageous in that it avoids sequential or co-transfection of two different vectors. However, it generates large, cumbersome vectors that are more difficult to manipulate. These vectors contain the cDNA encoding human κ L chain and $\gamma1$, $\gamma2$, $\gamma3$, $\gamma4$, or $\alpha1$ H chains. The expression of the Ig genes is under the control of the CMV promoter and the vectors contain cloning sites for V regions generated by PCR (Preston et al., 1998).

4. Production of IgG

The use of rodent Abs in humans is problematic because they are immunogenic, rapidly clear from circulation, and do not interact with human effector cells. Human MAbs are difficult to produce because cell lines are unstable and frequently produce Abs of the IgM isotype. In addition, there is only a limited range of human Ab specificities. However, with advances in genetic engineering, Abs can be generated which have increased affinity or avidity and with novel functional properties by fusing the Ab with non-Ig sequences.

Several different approaches have been used to generate MAbs with the desired specificity, reduced immunogenicity and the isotype appropriate for the desired effector functions. In phage display technology, combinatorial libraries containing large collections of V regions from naïve or immunized animals or from synthetic Ab genes are generated (reviewed in Hoogenboom et al., 1998). The V regions in the form of single chain Fvs or Fabs are displayed on the surface of filamentous phage particles by fusing them to one of the phage coat proteins. Antigen-specific phage Abs are then enriched by multiple rounds of affinity selection. The in vitro affinity-matured V regions are subsequently cloned into expression vectors containing C regions, creating functional Ab molecules.

XenoMouse technology is a powerful approach for the generation of fully human MAbs with high affinity

in mice. The murine Ig H and κ L chain loci are replaced with those of human on yeast artificial chromosome transgenes. The transgenes contain the majority of the human V region repertoire and the genes for Cκ, Cμ, Cδ and either Cγ1, Cγ2, or Cγ4 and *cis* regulatory elements required for their function. These mice were shown to be able to generate human Abs against a broad array of antigens and undergo class switching as well as somatic hypermutation and affinity maturation (Mendez et al., 1997).

Mouse/human chimeric Abs have been produced in which a murine V region is joined to the human C region domains (Boulianne et al., 1984; Morrison et al., 1984; Neuberger et al., 1985) or by grafting the antigen-binding sites of rodent Abs onto those of human Abs (Riechmann et al., 1988; Co and Queen, 1991). Chimeric Abs have been shown to retain their ability to bind antigen and display effector functions such as CDC, ADCC, and FcR binding characteristic of the human isotype (Boulianne et al., 1984; Morrison et al., 1984; Neuberger et al., 1984; Brüggemann et al., 1987; Dangl et al., 1988; Better and Horwitz, 1989; Canfield and Morrison, 1991).

MAbs represents one of the largest classes of drugs in development. Several recombinant Abs produced in myeloma cells have been approved for human therapies (Table 1) and many more are in late stages of clinical trials. MAbs are currently being used in the treatment of cardiovascular disease (Coller et al., 1996), infectious disease (Storch, 1998), inflammation (Present et al., 1999), cancer (Adkins and Spencer, 1998) and in transplantation (Waldmann and O'Shea, 1998; Kahan et al., 1999; Nashan et al., 1999). MAbs produced in CHO cells have also been used for treatment of cancer (Leget and Czuczman, 1998; Goldenberg, 1999; Flynn and Byrd, 2000).

Table 1
FDA approved MAbs produced in myeloma cells

Product name	Drug name	Indication	Reference
ReoPro	abciximab	Cardiovascular disease	Coller et al., 1996
Panorex	edrecolomab	Colorectal cancer	Adkins and Spencer, 1998
Remicade	infliximab	Inflammation	Present et al., 1999
Synagis	palivizumab	Respiratory syncytial virus infection	Storch, 1998
Zenapax	daclizumab	Transplant rejection	Waldmann and O'Shea, 1998
Simulect	basiliximab	Transplant rejection	Kahan et al., 1999

4.1. Mutational analysis of IgG

Because of the domain nature of Ig genes, it has been possible to delete or exchange exons between Ab molecules. Systematic comparisons of isotype-specific functions in the context of identical specificities have been made using mutational analysis as well as C region domain exchanged Abs. These studies have shown that the lower hinge region (residues 233–239) and the hinge-proximal bend (residues 327–331) between the two β-strands in C_H2 of IgG are critical for interacting with FcγRI (Duncan et al., 1988). Recently, an extensive analysis of the FcγR binding sites on human IgG1 has been reported in which all exposed amino acids in C_H2 and C_H3 were mutated individually (Shields et al., 2001). This comprehensive study revealed a set of IgG1 residues in C_H2 near the hinge (Glu233, Leu235, Gly236, Pro238, Asp265, Asn297, Ala327, Pro329) that are involved in binding to all human FcγRs. This set makes up the entire FcγRI binding site while other residues in C_H2 and C_H3 of IgG1 are also involved in binding to FcγRII and FcγRIII. Using this approach, other classes of mutants identified the amino acid residues that improve, reduce or have no effect on binding to FcγRII and/or FcγRIIIA. In addition, an enhancement in ADCC was demonstrated for select IgG1 variants with improved binding to FcγRIIIA. No residues were identified that only affected FcγRI binding.

Mutants derived from this study were also tested for binding to human FcRn. FcRn is involved in the transport of maternal IgG across the neonatal intestine of suckling rodents (Simister and Mostov, 1989; Zijlstra et al., 1990; Story et al., 1994; Leach et al., 1996; Simister et al., 1996). FcRn is also thought to be the salvage receptor for IgG, protecting IgG molecules from degradation in the lysosomal compartment (Ghetie et al., 1996; Israel et al., 1996; Junghans and Anderson, 1996). IgG binds to FcRn at pH 6.0 but not at pH 7.4 (Simister and Mostov, 1989; Ahouse et al., 1993). Variants containing single mutations were found that improved binding to FcRn as well as those that abrogated binding. IgG1 proteins

containing two (Glu380Ala/Asn434Ala) or three mutations (Thr307Ala/ Glu380Ala/Asn434Ala) were able to bind FcRn 8- or 11.8-fold better than wild-type IgG1 at pH 6.0 (Shields et al., 2001). These mutations may be useful in altering the half-life of therapeutic Abs. The crystal structures of FcγRIIIA:IgG and FcRn:IgG have been solved (Burmeister et al., 1994; Sondermann et al., 2000). However, this study demonstrated that residues outside of the Fc:receptor interface are critical for binding and biologic activity.

The C_H2 domain of IgG is involved in complement activation (Duncan and Winter, 1988; Gillies and Wesolowski, 1990). The core of C1q binding on human IgG1 has been mapped to residues Asp270, Lys322, Pro329 and Pro331, which are close together in three-dimensional space. Substitution mutations to any of these residues resulted in significant decreases in C1q binding and complement activation (Idusogie et al., 2000). However, other residues such as Leu235 and Asp265 are also involved in CDC (Morgan et al., 1995; Idusogie et al., 2000). In addition, mutation of residues Lys326 and Glu333 resulted in increases to C1q binding for human IgG1 while conferring the ability to bind C1q and fix complement on IgG2, which normally is inactive (Idusogie et al., 2000). The inability of IgG4 to activate complement results in part from the fact that it contains a Ser residue at position 331 while the other IgGs contain a Pro (Tao et al., 1993; Xu et al., 1994).

While most Ig isotypes are secreted as monomers, both IgM and IgA possess an 18 amino acid extension of the C terminus (tail-piece) which allows these Igs to polymerize. The addition of the IgA or IgM tail-pieces to IgGs results in the formation of IgG polymers (Smith and Morrison, 1994; Smith et al., 1995; Yoo et al., 1999; Sørensen et al., 2000). Polymerization not only increases the avidity of Igs for antigen, but also enhances effector functions such as complement activation, and binding to FcR (Smith and Morrison, 1994; Smith et al., 1995).

5. Antibodies as glycoproteins

All Abs are glycoproteins containing at least one N-linked carbohydrate attached to their H chain. Protein sequence determines the site of glycosylation with N-linked oligosaccharides attached by an N-glycosidic bond to Asn residues within the tripeptide Asn-X-Ser/Thr, with X being any amino acid except Pro. O-linked glycosylation of Abs occurs through the hydroxyl group of Ser or Thr (Opdenakker et al., 1993; Dwek et al., 1995; Snow and Hart, 1998) but no consensus sequence determining O-linked glycosylation is known. IgG has an N-glycosylation site at Asn297 in the C_H2 domain. The presence of carbohydrate in the C_H2 domain of IgG has been shown to be critical for engagement, through FcR binding, of phagocytic cells (Nose et al., 1983; Leatherbarrow et al., 1985; Tao and Morrison, 1989). Aglycosylated IgG is also impaired in its ability to carry out CDC (Nose et al., 1983; Leatherbarrow et al., 1985; Tao and Morrison, 1989; Dorai et al., 1991). The absence of C_H2-associated carbohydrate is thought to cause conformational changes in the C_H2 and hinge regions which result in loss of function (Lund et al., 1993b). In most cases, aglycosylation had little effect on the serum half-life and biodistribution of Abs in mice (Tao and Morrison, 1989; Dorai et al., 1991) and in primates (Hand et al., 1992). However, Ab–Ab complexes produced from carbohydrate-deficient Abs failed to be eliminated rapidly from the circulation (Nose et al., 1983).

5.1. Carbohydrate processing

Carbohydrate is added co-translationally to the growing polypeptide chain. A preformed moiety of $mannose_9$ $glucose_3$ *N*-$acetylglucosamine_2$ is transferred from a dolichol intermediate. The terminal glucoses are bound by the chaperone calnexin and must be removed to allow transit through the endoplasmic reticulum. The processing steps are shown schematically in Fig. 3. Analysis of carbohydrates isolated from normal human serum IgG has yielded up to thirty different structures (Rudd et al., 1991) with structural differences resulting from differences in core substitution of fucose and/or bisecting *N*-acetylglucosamine (GlcNAc) and in processing of the outer arms of the biantennary sugar as indicated in the final product in Fig. 3. Mouse cells can add an additional terminal galactose with a novel α1,3 linkage (Weitzhandler et al., 1994; Sheeley et al., 1997). This residue is strongly immunogenic in humans and over 1% of serum IgG is directed against the Galα1,3-Galβ1,4-GlcNAc epitope, possibly as a consequence of its presence on

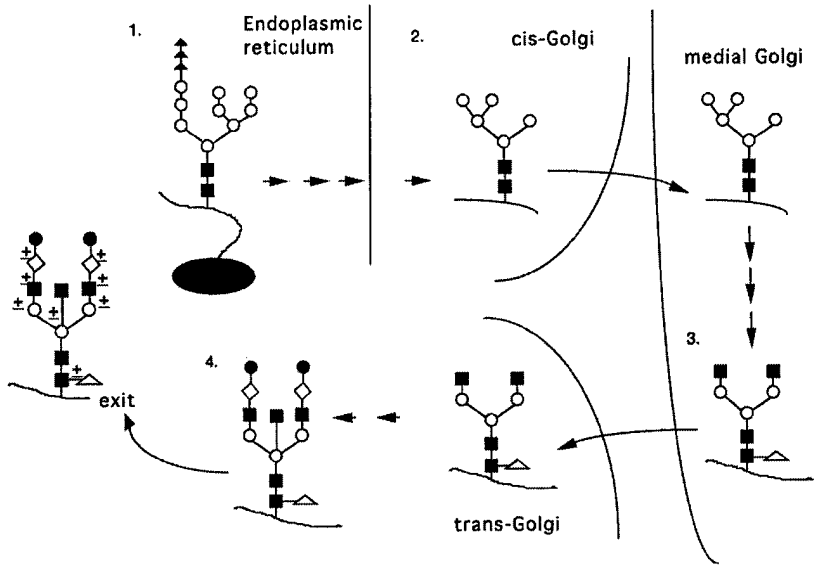

Fig. 3. An abbreviated schematic representation of the processing pathway of oligosaccharide to complex biantennary form (Kornfeld et al., 1985). The newly synthesized species $Glu_3Man_9GlcNAc_2$ (1) is transferred from dolichyldiphosphate to the Asn-X-Ser/Thr sequence in the peptide as it emerges from the ribosome. The arrows indicate sequential enzymatic reactions through which sugar residues are trimmed as the glycoprotein passes through the endoplasmic reticulum. After removal of the three terminal glucoses, the glycoprotein moves to the *cis*-Golgi, where it undergoes a series of steps through which mannose residues are trimmed by α-mannosidases. Processing can stop at this point yielding glycoproteins with high mannose sugars attached. Alternatively, processing can proceed to yield $Man_5GlcNAc_2$ (2). This intermediate is the preferred substrate for *N*-acetylglucosaminyltransferase I whose action, in the medial Golgi, is the committed step in complex oligosaccharide synthesis. The CHO glycosylation mutant Lec1 is deficient in this enzyme so the sugars produced by these cells bear this structure. In the medial (3) and *trans*-Golgi (4) the oligosaccharide undergoes further processing steps in which mannose residues are trimmed and the sugar residues are sequentially added. The newly synthesized glycoprotein then exits the Golgi and is transported to the cell membrane or is secreted. (±) indicates that the final carbohydrate structure may or may not contain the particular sugar residue. Symbols: glucose (▲); mannose (○); *N*-acetylglucosamine (■); fucose (△); galactose (◇); sialic acid (●).

enteric bacteria (Hamadeh et al., 1992). CHO cells as well as human, ape, and Old World monkey cells lack the enzyme required to attach the α1,3 galactosyl structure (Borrebaeck et al., 1993).

5.2. Structure

As noted above, heterogeneity in processing of the final biantennary carbohydrate results in the attachment of carbohydrates of differing structures (Mizuochi et al., 1982). A growing body of evidence suggests that certain alterations in carbohydrate structure can affect Ab function. A link between the agalactosylated Abs and disease has been suggested by several studies (Parekh et al., 1985; Rademacher et al., 1988, 1994). It has been proposed that agalactosyl IgG antibodies may contribute to inflammation through binding of mannose binding protein (MBP; Malhotra et al., 1995). MBP contains carbohydrate recognition domains that recognize terminal fucose, mannose, glucose, and GlcNAc but not galactose and bind preferentially, but not uniquely, to agalactosyl Abs (Wright and Morrison, 1998). MBP bears structural similarities to C1q and through binding to Fc regions initiates complement activation. Degalactosylated IgG and Fc fragments incubated with MBP show enhanced deposition of complement component C4b compared with untreated Abs (Malhotra et al., 1995).

Carbohydrate has also been suggested to play a role in glycoprotein targeting and clearance (reviewed in Drickamer et al., 1993). The mammalian asialoglycoprotein receptor specific for galactose and *N*-acetylgalactosamine is found on hepatocytes and mediates clearance of proteins with exposed terminal galactose

(N-linked) or *N*-acetylgalactosamine (O-linked). The mannose receptor of macrophages and hepatic endothelial cells mediates binding and clearance of glycoconjugates terminating in mannose, fucose or GlcNAc. Glycoproteins bound to either receptor are internalized and transported to lysosomes for degradation. A fucose- and galactose-specific receptor is found on Kupffer cells, the resident macrophages of the liver.

5.3. Variable region glycosylation

Human serum IgG has on average 2.8 N-glycoside-type sugar chains per protein molecule (Kinoshita et al., 1991). Two of these carbohydrate moieties belong to the conserved N-linked carbohydrate in the Fc region with the remainder reflecting V region glycosylation. The N-linked sugar chains of the Fab moiety of IgG can influence Ab aggregation and stability. The cryoglobulin and cold agglutinin properties of certain monoclonal IgG and IgM molecules have been shown to arise from sialylated N-linked sugar located on the Fab (Hymes et al., 1979; Middaugh and Litman, 1987; Kinoshita et al., 1991). Aggregated IgG isolated from human plasma carries more oligosaccharide chains than monomeric IgG (3.8 and 2.2, respectively) with an increased level of Fab-associated disialylated structures (Parekh et al., 1988). In several cases, it has now been demonstrated that differences in V region glycosylation can influence both the affinity and specificity of Abs (Tachibana et al., 1992; Co et al., 1993; Kato et al., 1993; Kusakabe et al., 1994). Glycosylation of Asn58 in V_H CDR2 of an anti-dextran Ab was shown to increase the affinity of the Ab for antigen 10-fold (Wallick et al., 1988). Analysis of the structure of the carbohydrate attached at Asn58 following H chain expression in a H chain loss variant of a murine hybridoma revealed complex type sugar chains like the Fc carbohydrate. However, unlike the Fc associated carbohydrate, a portion of the sugar chains on Asn58 contained the Galα1 $\rightarrow$ 3Gal groups as a nonreducing terminus. In addition, the complex biantennary sugar chains on the V region were more highly sialyated than those on the C region.

5.4. Expression systems and glycosylation

Given the contribution of carbohydrate structure to protein function, it becomes important to know what controls the structure of the attached carbohydrate. Certain mouse lines such as hybridomas and mouse–human heterohybridomas synthesize glycans terminating in Galα1,3–Galβ1,4–GlcNAc (Borrebaeck et al., 1993) particularly when grown in nonagitated flasks (Lund et al., 1993a). But other rodent lines such as mouse NSO or rat Y3 myelomas producing humanized Abs do not add Galα1,3-Galβ1,4-GlcNAc (Lifely et al., 1995). *N*-glycolylneuramic acid (NeuGc), a derivative of *N*-acetylneuraminic acid (NeuAc), has been shown to be more prevalent than NeuAc in Abs from mouse or human–mouse hybridomas (Monica et al., 1995; Leibiger et al., 1998). Proteins from human adults do not normally contain NeuGc, which is an oncofetal antigen. In general, mouse–human heterohybridomas follow the glycosylation pattern characteristic of the mouse parental line (Monica et al., 1995; Leibiger et al., 1998). A significant proportion of IgG molecules produced by human B lymphocytes possess a bisecting GlcNAc residue β1-4 linked to the central β-linked mannose of the core glycan. Presence of this residue appears to enhance the ability of IgG to mediated ADCC (Umana et al., 1999). Only certain rodent cell lines such as the rat Y3 myeloma (but not CHO or NSO) produce recombinant Abs containing this bisecting residue (Lifely et al., 1995). External conditions can also influence the structure of the attached carbohydrate. IgG produced by mouse hybridomas in serum-free medium has higher levels of terminal sialic acid and galactose residues relative to that produced using serum. The ambient glucose concentrations have been found to affect the degree of glycosylation of MAbs produced by human hybridomas in batch culture. Therefore, cell culture conditions can influence both the extent and structure of the carbohydrate on Abs produced in myeloma cell lines (reviewed in Jenkins et al., 1996).

6. IgA

In humans, the synthesis of IgA exceeds the combined total of all the other Ig classes (Conley and Delacroix, 1987; Childers et al., 1989). While functions of serum IgA are not understood, IgA in external secretions neutralizes toxin, agglutinates bacteria and binds virus thus preventing them from attaching to the mucosal surfaces of the respiratory, gastrointestinal

and the genito-urinary tract (Russell et al., 1999). If infectious microorganisms, such as viruses, get past the first line of defense and succeed in infecting the mucosal epithelium, specific IgA in the process of transcytosis may neutralize the virus (Mazanec et al., 1993). Furthermore, if the pathogen or antigen is found in the lamina propia, specific IgA can bind to form immune complexes that are transported across epithelial cells from the basal to the apical surface by polyimmunoglobulin receptor (pIgR)-mediated transport. Because of the importance of IgA at the mucosal surfaces, there is considerable interest in developing oral and intranasal therapeutics based on IgA.

6.1. IgA structure

Human IgA exists as two isotypes, IgA1 and IgA2. Three allotypes of IgA2 have been described: IgA2m(1), IgA2m(2) and IgA2(*n*) (Mestecky and McGhee, 1987; Kerr, 1990; Chintalacharuvu et al., 1994). The major difference between the IgA1 and IgA2 subclasses is a 13 amino acid deletion in the IgA2 hinge region. A striking characteristic of IgA is its presence as different molecular forms with a characteristic distribution in various body fluids (Kaartinen et al., 1978; Delacroix et al., 1982, 1983). The predominate form of IgA in the serum is monomeric, with a H_2L_2 structure, although smaller amounts of dimer, trimer and tetramer are also present. An 18 amino acid extension found at the C-terminus of Cα3 contains a penultimate Cys required for polymer formation. Polymeric IgA (pIgA) consists of multiple H_2L_2 building blocks covalently linked through the J chain protein (Koshland, 1985). Like H and L chain, J chain is a product of the plasma cell. In dimeric IgA, the J chain is disulfide linked to each monomer through one of the penultimate Cys. The production of pIgA requires the expression of three proteins—H, L and J chain. When a myeloma expression system is used, the endogenous myeloma J chain is incorporated into the IgA polymers (Chintalacharuvu and Morrison, 1996). Therefore, to produce pIgA in myeloma cells, only H and L chain must be transfected. In contrast, CHO cells require the transfection of H, L and J chain for the expression of pIgA. If the goal is the production of fully human pIgA, a potential shortcoming of using a murine myeloma for expression is that the J chain will be murine, which differs at 32 of 137 amino acids from that of human (Johansen et al., 2000).

Secretory IgA (sIgA), found in external secretions, is always polymeric and linked to a 80 kD protein known as secretory component (SC) or the ectoplasmic domain of the pIgR (Tomasi et al., 1965; Mostov, 1994). sIgA is unusual in that it is the product of two cell types, the plasma cell and the epithelial cell, and contains four different polypeptide chains: αH chain, L chain, J chain and SC. Co-culture systems using hybridomas and polarized monolayers of epithelial cells and in vitro mixing of purified pIgA and SC have been used to produce small quantities of sIgA. However, when murine transfectomas secreting chimeric IgA1 were transfected with a SC expression vector, cells lines were isolated that expressed SC with virtually all of the SC secreted covalently associated with IgA (Chintalacharuvu and Morrison, 1997). Pulse-chase experiments suggested that SC is covalently linked to IgA intracellularly just prior to the time of secretion. In the parental cell line, chimeric IgA1 dimerizes late in the secretory pathway presumably when J chain is incorporated into the molecule (Chintalacharuvu and Morrison, 1996) and it is possible that the assembly of sIgA in the transfected myeloma cells takes place in the Golgi apparatus when pIgA and SC are present together (Chintalacharuvu and Morrison, 1997). sIgA has also been assembled in CHO cells by transfecting with expression vectors coding for the αH chain, κL chain, J chain and SC (Berdoz et al., 1999; Johansen et al., 1999). Secretory IgA assembled in single cell systems binds antigen and shows increased stability to intestinal proteases in vitro (Chintalacharuvu and Morrison, 1997; Berdoz et al., 1999).

6.2. Role of carbohydrates in IgA

In addition to the heterogeneity of the polypeptide composition, IgA is also a highly glycosylated molecule. Depending on the isotype of IgA there are two to five N-linked carbohydrates attached to each α chain and one N-linked carbohydrate attached to the J chain. In sIgA, one to seven additional N-linked carbohydrates are attached to SC (Piskurich et al., 1995). There are up to five O-linked carbohydrates in the hinge region of each α chain in IgA1, which is a substrate for a number of bacterial proteases (Kilian and Russell, 1999). More than 90% of N-linked carbohydrates in

recombinant IgA1 are sialated with mostly biantennary structures in C_H2 and triantennary structures in C_H3 (Mattu et al., 1998). In addition, the glycosylation on IgA produced by murine B cells has been shown to be influenced by the presence of cytokines (Chintalacharuvu and Emancipator, 1997). Molecular modeling suggested that the N-glycans in IgA1 Fc are not confined within the inter-H chain space but are instead accessible on the surface. The hydrophilic carbohydrates are expected to impart unique physiochemical properties to sIgA in the hostile environment of the respiratory, gastrointestinal and genito-urinary tract.

Deletion of one or both N-linked carbohydrates did not interfere with synthesis and secretion of human IgA1 (Chuang and Morrison, 1997) or affect its ability to bind the pIgR or neutrophil Fcα receptor (Mattu et al., 1998) but did interfere with murine IgA secretion (Taylor and Wall, 1988). However, deletion of the N-glycan in C_H3 alone or in C_H2 and C_H3 increased the percentage of IgA found as trimers and tetramers (Chuang and Morrison, 1997). Although IgA lacking N-linked carbohydrate in the C_H3 domain showed a reduced ability to bind to complement component C3, none of the IgA1 proteins appeared to activate the alternative pathway.

When recombinant human IgA1 and IgA2 produced in Sp2/0 cells were injected intravenously into C57Bl/6 mice, all three allotypes of IgA2 Abs were removed from the blood by the liver more rapidly than IgA1 (Rifai et al., 2000). All three allotypes of IgA2 cleared more slowly in C57Bl/6 mice in the presence of galactose-Ficoll conjugate and in asialoglycoprotein-binding receptor (ASGR)-deficient mice, indicating that ASGR is responsible for the rapid removal of IgA2 from blood. Carbohydrate also plays a role in the long-term clearance of IgA1. It is proposed that IgA1 with under galactosylated O-linked carbohydrates may be responsible for the deposition of IgA in the mesangium of patients with IgA nephropathy (Emancipator et al., 1999). In addition, IgA1 is cleared more slowly in ASGR-deficient mice than in wild type mice and IgA1 lacking N-linked carbohydrate cleared significantly slower than wild type IgA1. However, IgA1 lacking the hinge with its associated O-linked carbohydrate was cleared more rapidly than wild type IgA1 (Rifai et al., 2000). These results suggest that dysfunction of the ASGR and/or aberrant N-linked glycosylation of IgA may account for the elevated serum IgA in liver diseases and IgA nephropathy.

7. Antibody fusion proteins

There has been rapid progress in the development of Abs fused to other proteins. Ab fusion proteins, also known as immunoligands (Penichet et al., 1999b), retain the ability to bind antigen while the attached ligand is able to bind its respective receptor. In addition, if the Fc fragment is preserved, the fusion protein also retains Ab effector functions. Ab fusion proteins can be produced using several different strategies (Fig. 4). When the non-Ab partner is fused to the end of the C_H3 domain (C_H3-ligand), the Ab combining specificity can be used to provide specific delivery of an associated biologic activity as well as Fc effector functions. Immunoligands with the ligand fused immediately after hinge (H-ligand) or to the

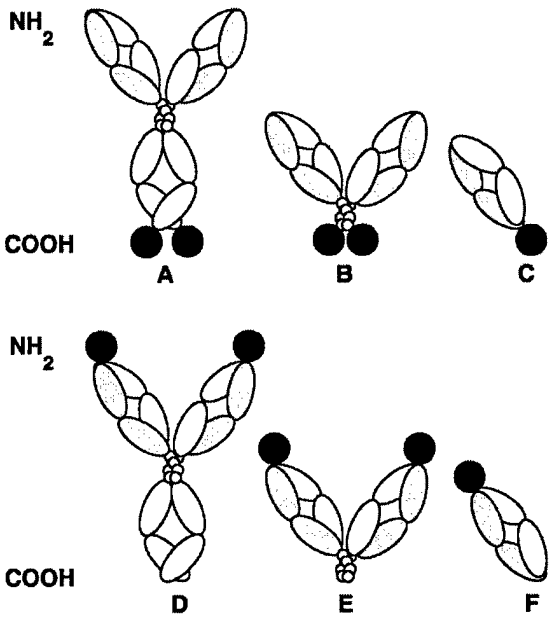

Fig. 4. A schematic diagram of immunoligands. Ab-fusion proteins in which the ligand is fused to the C-terminus after the C_H3 domain (A), immediately after hinge (B), or after the C_H1 domain (C). Alternatively, the ligand can be joined to the N-terminus of the full-length (D) or truncated H chain (E and F).

C_H1 domain (C_H1-ligand) may be useful when the Fc-related effector functions are unnecessary or harmful. In addition, for many applications the small size of the H-ligand and C_H1-ligand may be advantageous over the larger C_H3-ligand. An alternative approach is to construct Ab fusion proteins with the ligand fused to the N-terminus of the H chain (Fig. 4). This may be necessary for proteins which require N-terminal processing or proper folding for activity such as nerve growth factor (McGrath et al., 1997), the co-stimulatory molecule B7.1 (Challita-Eid et al., 1998) and interleukin 12 (IL12) (Peng et al., 1999).

7.1. Antibody fusion proteins for the treatment of cancer

Despite considerable advancement in cancer therapy, relapse is still a major problem in the clinical management of cancer. Chemotherapeutic strategies are limited by toxicity and poor efficacy. Therefore, additional modalities are needed to achieve disease containment or elimination. Systemic treatment with cytokines such as IL2, IL12 and granulocyte-macrophage colony-stimulating factor (GM-CSF) can render some non-immunogenic tumors immunogenic, activating a protective immune response (Ruef and Coleman, 1990; Tsung et al., 1997; Rosenberg et al., 1998). However, when cytokines are given systemically there are frequently problems with severe toxic side effects that make it impossible to achieve an effective dose at the site of the tumor (Siegel and Puri, 1991; Maas et al., 1993; Cohen, 1995). Tumor specific Abs genetically fused to cytokines provide an alternative approach for concentrating in the region of the tumors quantities of cytokine sufficient to elicit a significant anti-tumor activity without accompanying systemic toxicity. Using the myeloma expression system, we and others have successfully developed several Ab-cytokine fusion proteins specific for different tumor associate antigens (TAAs).

Ab-IL2 fusion proteins have been the best characterized and most broadly used in successful anti-tumor experiments using animal models (Penichet and Morrison, 2001). The first tumor specific Ab-IL2 fusion protein that we developed was a human IgG3 specific for the idiotype (Id) of the Ig expressed on the surface of the B cell lymphoma 38C13 with human IL2 fused at the end of the C_H3 domain (Penichet et al., 1998). This Ab fusion protein (IgG3-C_H3-IL2) expressed in Sp2/0 was properly assembled and secreted. Anti-Id IgG3-C_H3-IL2 has a half-life in mice of approximately 8 h, which is 17-fold longer than the half-life reported for IL2, and it showed a better localization of subcutaneous tumors in mice than the anti-Id IgG3. Most importantly, the anti-Id IgG3-C_H3-IL2 showed enhanced anti-tumor activity compared to the combination of Ab and IL2 administered together (Table 2; Penichet et al., 1998). In addition, a chimeric anti-Id IgG1-IL2 fusion protein (chS5A8-IL2) expressed in P3X63Ag8.653 was more effective in the in vivo eradication of the 38C13 tumor than the combination of the anti-Id Ab and IL2 or an Ab-IL2 fusion protein with an irrelevant specificity (Liu et al., 1998).

Remarkable success in pre-clinical trials has been obtained using a chimeric anti-GD_2 IgG1-IL2 fusion protein (ch14.18-IL2) produced in Sp2/0 cells (Becker et al., 1996a,b,c). ch14.18-IL2 treatment of mice bearing pulmonary and hepatic metastases as well as subcutaneous GD_2 expressing B16 melanoma resulted in a specific and strong anti-tumor activity. This anti-tumor activity was significant compared to Ab (ch14.18) and IL2 or irrelevant Ab-IL2 fusion proteins and resulted in the complete eradication of the tumor in

Table 2
Results of in vivo therapy experiments

Group	Treatment	Disease free survivors[a]	
		Experiment 1[b]	Experiment 2[c]
1	PBS	0/6 (0%)	0/8 (0%)
2	anti-Id IgG3	1/6 (16.7%)	2/8 (25%)
3	IL2	0/6 (0%)	0/8 (0%)
4	anti-Id IgG3 + IL2	0/6 (0%)	4/8 (50%)
5	anti-Id IgG3-IL2	3/6 (50%)	7/8 (87.5%)
6	anti-dansyl IgG3-IL2	not done	2/8 (25%)

[a] Animals surviving 60 days without evidence of tumor were considered to be tumor free.

[b] Groups of 6 C_3H/HeN mice were injected i.p. with 1000 38C13 cells. The following day, each group received single i.p. injections of PBS, 10 μg of anti-Id IgG3, 30,000 IU of IL-2, both 10 μg anti-Id IgG3 and 30,000 IU of IL-2, or 10 μg of anti-Id IgG3-IL2.

[c] Groups of 8 C_3H/HeN mice were injected s.c. with 1000 38C13 cells. The following day, each group received the first of five daily i.p. injections. Groups were treated with PBS, 10 μg of anti-Id IgG3, 30,000 IU of IL-2, both 10 μg anti-Id IgG3 and 30,000 IU of IL-2, 10 μg of anti-Id IgG3-IL2 or 10 μg of anti-dansyl IgG3-IL2, which contains an irrelevant specificity.

a vast number of animals (Becker et al., 1996a,b,c). Similar results have been obtained in mice bearing CT26-KSA hepatic and pulmonary metastases and treated with a humanized anti-KSA Ab-IL2 fusion protein (huKS1/4-IL2) produced in NSO (Xiang et al., 1997, 1999).

The successful use of the myeloma expression system for the production of Ab fusion proteins specific for TAAs has led to a significant expansion of the anti-tumor Ab-cytokine fusion proteins. Other examples of these novel molecules are a chimeric anti-human MHC class II IgG1 fused to GM-CSF (chCLL-1/GM-CSF) expressed in NSO (Hornick et al., 1997), and a humanized anti-HER2/*neu* IgG3 fused to IL12 or GM-CSF expressed in P3X63Ag8.653 (Peng et al., 1999; Dela Cruz et al., 2000).

7.2. Antibody fusion proteins for brain targeting

One region of the body particularly difficult to target is the brain due to the presence of the blood–brain barrier (BBB). This highly resistance barrier, which maintains homeostasis within the brain, is formed by tightly joined capillary endothelial cell membranes (Brightman and Tao-Cheng, 1993; Abbott and Romero, 1996). The BBB effectively restricts transport from the blood of certain molecules, especially those that are water soluble and larger than several hundred daltons (Shapiro and Shapiro, 1986), limiting the clinical utility of many proteins of diagnostic and/or therapeutic interest for the brain. However, the BBB has been shown to have specific receptors which allow the transport of several macromolecules such as insulin (Duffy and Pardridge, 1987), transferrin (Tf; Fishman et al., 1987), and insulin-like growth factors 1 and 2 (IGF1 and IGF2; Rosenfeld et al., 1987) from the blood to the brain.

One approach for Ab brain targeting is the fusion of the Ab of interest to one of the molecules with receptors on the BBB or the development of Abs specific for such receptors. In an initial attempt, we developed Ab fusion proteins by fusing IGF1, IGF2 (Shin et al., 1994) or Tf (Shin et al., 1995) to chimeric IgG3 at the end of the C_H1 domain, immediately after the hinge, and at the end of the C_H3 domain. All of these molecules expressed in murine myeloma cell lines showed significant uptake into the brain parenchyma (Shin et al., 1994, 1995). These Ab fusion proteins, which are specific for the hapten dansyl, can serve as "universal vectors" for the delivery of any dansylated molecule to the brain.

Table 3
Brain uptakes of biotin-PNA with or without anti-TfR IgG3-Av[a]

Injectate	Brain uptake (%ID/g brain)
[^{125}I]-Biotin-PNA	0.0083 ± 0.0009
anti-TfR IgG3-Av+[^{125}I]-Biotin-PNA	0.12 ± 0.03

[a] Measurements were made 60 min after i.v. injection of 5 μCi (0.1 nmol) of [^{125}I]-Biotin-PNA alone or conjugated with 0.1 nmol of anti-TfR IgG3-Av. Data are mean ± SE ($n = 3$, rats). Av, avidin; ID, injected dose; PNA, peptide-nucleic acid.

Another strategy for developing a universal delivery vehicle is to exploit the broadly used avidin–biotin technology. An Ab specific for the transferrin receptor (TfR) was genetically fused to avidin (Av). Anti-TfR IgG3-C_H3-Av exhibited both Ab- and avidin-related activities (Penichet et al., 1999a). This fusion protein was able to deliver [^{3}H]biotin and a biotinylated antisense oligonucleotide complementary to the *rev* gene of HIV-1 to the brain. Brain uptake of the HIV antisense drug was increased at least 15-fold when it was bound to the anti-TfR IgG3-CH3-Av, suggesting its potential use in neurological AIDS (Table 3; Penichet et al., 1999a). This novel Ab fusion protein should have general utility as a universal vehicle to effectively deliver biotinylated compounds across the BBB for the diagnosis and/or therapy of a broad range of brain disorders such as infectious diseases, brain tumors, Parkinson's disease and Huntington's disease.

8. Conclusion

Myeloma expression systems has been successfully used for the production of MAbs for both research and commercial applications. The use of plasmid vectors containing the Ig regulatory elements or a heterologous promoter and enhancer have been used to produce Abs in relatively large quantities. Strategies to isolate murine and human V regions by PCR and through phage display techniques have been extremely successful, making it possible to produce Abs with almost any desired specificity. Chimeric Abs

that contain murine V regions and human C regions have been produced in myeloma expression systems. These Abs retain both their specificity and effector functions while reducing immunogenicity in humans.

Chimeric Abs with gene segments derived from diverse sources can easily be generated. Since genes can be modified before they are expressed, C regions with improved biologic properties can be produced. Studies using variants with point mutations or ones in which domains are exchanged or deleted have been helpful in delineating which amino acid residues and structural motifs are involved in contributing to Ab function.

Antibodies are hetero-multimers that must be covalently assembled and post-translationally modified by glycosylation. Myeloma expression systems effectively assemble and secrete the H_2L_2 multimer that is characteristic of IgG as well as the higher polymeric forms of IgA. In addition, myelomas expressing SC along with IgA produce sIgA, which is normally the product of two different cell types. Numerous studies indicate that glycosylation contributes to the proper function of Abs. As in human serum, the MAbs produced in myeloma cells display significant heterogeneity in glycosylation with variability in site usage and in processing dependent on the species in which the myeloma arose, cellular variations and growth conditions.

Fusion proteins with intact Ab or Ab fragments fused to non-Ig sequences have been shown to be multifunctional, retaining the ability to bind antigen, Ab effector functions and the activity of the non-Ab partner. Ab fusion proteins have been used successfully in treating cancers and in targeting to the brain in animal models.

Abs are ideal molecules for diagnostic and therapeutic applications for several reasons—they have exquisite specificity for a given target, they are robust molecules that are amenable to genetic manipulation, they can be produced with relative ease, and their structure and function have been studied extensively. The knowledge gained from studies using Abs and Ab variants should aid in rationally designing Abs so that they contain the combination of characteristics most appropriate for a given application. Myeloma cell lines are an excellent system for the production of recombinant Abs as evidenced by the many now available in the clinic.

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Appendix 8

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procedure consists of subcutaneous injections of 0.5 mL at 0, 2, and 4 weeks, 6, 12, and 18 months, and subsequent annual injections. Side effects may include mild, local reactions consisting of erythema and tenderness; moderate, local inflammation; or systemic reactions (in approximately 0.5% of recipients) consisting of headache, fever, general body aches, and malaise. The vaccine is generally thought to be safe and effective.

USING AT TO DELIVER CYTOTOXIC T LYMPHOCYTE EPITOPES

The ability of PA to mediate cytosolic entry of heterologous proteins fused to LF_N has been exploited to develop a vaccine system directed against intracellular pathogens (2). Because intracellular pathogens have limited exposure to host humoral defences, the immune system attempts to deprive them of their growth niche by recognizing and lysing infected cells. Bacterial or viral proteins in the cell cytosol are degraded by the proteasome; certain peptides released by this process are transported into the endoplasmic reticulum in which they are complexed with major histocompatibility complex-1 (MHC-1) molecules. The complexed peptides are presented on the cell surface where they are recognized by cytotoxic T-cells (CTL). CTL lyse the infected cell, release cytokines, and expand. Some of the expanded CTL differentiate into memory CTL, which allow for an enhanced response on subsequent exposure. It is these memory CTL that vaccines are intended to elicit.

Fusions of LF_N to CTL epitopes derived from viral and bacterial pathogens stimulate epitope-specific CTL in a manner that depends on PA. Epitopes may be genetically fused to the amino-terminus or carboxy-terminus of LF_N, or linked by a disulfide bond. Furthermore, a single peptide containing two different epitopes has been shown to elicit a CTL response against multiple pathogens (3). The efficacy of the induced CTL response is demonstrated by the vaccine-induced protection of mice against bacterial and viral challenge. Advantages of this system include that it requires only small amounts of fusion protein and no adjuvant, it does not generate an antibody response to the vaccine components (allowing for multiple vaccinations), and it may be used to expand antigen-specific CTL in vitro (suggesting a potential use in disease therapy). Other uses of toxin fusion proteins could include delivering drugs that are otherwise unable to cross membranes. Futhermore, PA-mediated delivery of heterologous proteins could be used in research to alter signal transduction pathways or to introduce proteins that have been modified in vitro.

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The antibodies (Abs) are molecules with multiple properties that make them a critical component of the immune system. These properties include the ability to recognize a vast array of different molecules known as antigens (Ags) and to interact with and activate the host effector systems. The value of Abs with defined specificities is clear, and with the development of hybridoma technology, it was possible to produce murine monoclonal Abs that represent the product of a single clone of Ab-producing cells and have only one Ag-binding specificity (1). Advances in genetic engineering and expression systems have led to further improvements in the development of monoclonal Abs allowing the production of chimeric (2), humanized (2), and totally human Abs (3) as well as Abs with novel structures and functional properties (2,4,5). As consequence Ab-based therapies are now used for a variety of diverse conditions that include viral infections, inflammatory disorders, and cancer (6).

ANTIBODY STRUCTURE AND ENGINEERING

Abs are composed of light and heavy chains joined by disulfide linkages (Fig. 1). It is the heavy chain that determines the biological properties of the Ab and several classes, or isotypes of Abs with different heavy chains are found in mammals. The light and heavy chains fold into functional domains, two for the light chain, four or five for the heavy chain depending on the isotype. The N-terminal domain from each chain forms the variable regions, V_L and V_H, which constitute the Ag binding sites. The other domains contribute to the Ab effector functions. The domain structure of the Ab facilitates protein engineering allowing the exchange between molecules of functional domains carrying Ag-binding activities (Fabs or Fvs) or effector functions (Fc). It also makes it easy to produce bispecific Abs containing more than two Fab fragments (2,7) or Ab fusion proteins in which non-Ab molecules such as cytokines, growth factors, and enzymes are genetically fused to the Ab (2,4,8–12).

CHIMERIC, HUMANIZED AND HUMAN ANTIBODIES

The use of mouse monoclonal Abs in humans often results in a human antimouse antibody (HAMA) response and as consequence the murine Abs are rapidly cleared from circulation making the treatment ineffective (13). In addition, mouse constant regions can be ineffective in interacting with the human immune effector systems. To overcome these problems chimeric Abs with murine variable regions joined

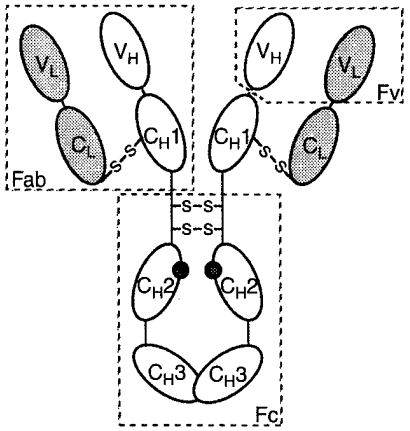

Figure 1. Schematic representation of an immunoglobulin G (IgG) molecule and the active fragments that can be derived from it. These fragments were originally defined by differential proteolytic cleavage, but are now commonly produced by genetic engineering. The carbohydrate units (black circles), present within the C_H2 domains contribute to the functional properties of the Ab.

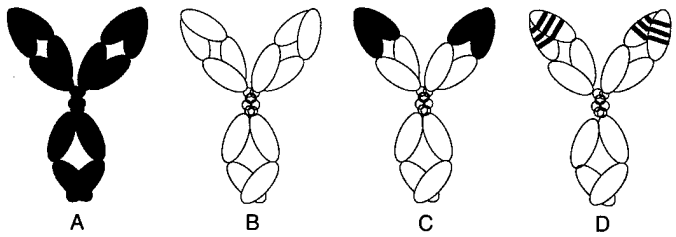

Figure 2. Schematic diagram of murine monoclonal Ab (A), human monoclonal Ab (B), mouse-human chimeric Ab, (C) and CDR-grafted or humanized Ab (D). Chimeric Abs have murine derived variable regions and binding specificities joined to human constant regions and effector functions. Humanized Abs are composed of mostly human sequences except those areas in contact with the Ag (CDR regions), which are derived from mouse sequences.

to human effector regions have been produced (2) (Fig. 2). For the most part, these Abs retain their target specificity and show reduced HAMA responses. An example of a successful mouse-human chimeric Ab is rituximab (Rituxan) which targets the CD20 Ag and is now widely used to treat lymphoma (14).

Although mouse-human chimeric Abs are less immunogenic than murine Abs in some cases they can still elicit a significant human antichimeric Ab (HACA) response (15). One approach to this problem is to further manipulate the Ab variable region encoding for the Ag binding site resulting in humanized Abs (2) (Fig. 2). The Ag binding site is formed from the six complementary-determining regions (CDRs) of the variable portion of the heavy and light chains. Each variable domain consists of 7 antiparallel β-strands connected by loops and forming a β-barrel. Among the loops are the CDR regions. It is feasible to move the CDRs and their associated specificity from one scaffolding β-barrel to another thereby creating "CDR-grafted" or "humanized" antibodies. However, it is rarely sufficient merely to move the CDRs from a murine Ab on to a completely human framework because the resulting Ab frequently has reduced or no binding activity. In these cases new constructs are made that incorporate additional murine residues near the CDRs until binding is restored. An example of a successful humanized Ab recently approved for clinical use is trastuzumab (Herceptin) which has demonstrated significant antitumor activity in patients affected with breast cancer and overexpressing the tumor-associated Ag HER2/*neu* (16).

It has recently been possible to make mice that produce Ag-specific antibodies that are totally human. This has been accomplished by inserting elements of the human heavy- and light-chain loci into mice in which production of endogenous murine heavy and light chains was disrupted (3). The resulting mice can synthesize human Abs specific for many Ags, including Ags of human origin and can be used to produce hybridomas making human Abs. To date these engineered mice can produce only some of the human isotypes, but further genetic modification of them promises to expand their potential.

ANTIBODY FUSION PROTEINS

Fusion proteins with non-Ab molecules fused to Abs can be produced using several different approaches. Ab fusion proteins that contain an intact Ag binding site should retain the ability to bind Ag whereas the attached ligand should be able bind its receptor. Such molecules, known as "immunoligands," can be produced in several different ways (Fig. 3, panels A-F). When the non-Ab partner is fused to the end of the C_H3 domain (C_H3-ligand) (Fig. 3, panel A) the Ab combining specificity can be used to provide specific delivery of an associated biological activity and Ab-related effector functions. Examples of these molecules are Abs targeting cancer cells fused with interleukin-2 (Ab-(IL2) fusion proteins) which have shown significant antitumor activity in mice bearing tumors (8,11,12) and an antitransferrin receptor Ab-avidin fusion protein which has been used successfully to deliver biotinylated compounds to the brain through the blood/brain barrier (17). Immunoligands with the ligand fused immediately after hinge (H-ligand) (Fig. 3, panel B) or to the C_H1 domain (C_H1-ligand) (Fig. 3, panel C) may be useful when the Ab-related effector functions are unnecessary or harmful. In addition, for many applications the small size of the H-ligand and C_H1-ligand may be an advantage over the larger C_H3-ligand.

An alternative approach is to construct Ab fusion proteins with the ligand fused to the N-terminus of the heavy chain (Fig. 3, panels D, E, and F). This may be necessary for proteins which require N-terminal processing or folding for activity such as nerve growth factor (NGF), the costimulatory molecule B7.1 and interleukin-12 (IL 12). In fact, Ab-NGF, Ab-(B7.1), and Ab-IL 12 fusion proteins containing the ligand fused to the N-terminus of Ab retain both the ability to bind Ag and the activity of the non-Ab partner (9,10,18).

Alternatively, non-Ab sequences can be used to replace the V_H domain or the V_H-C_H1 domains (Fig. 3, panels G and H). These molecules, which lack the ability to bind Ag, have been called "immunoadhesins" because they contain an adhesive molecule linked to the immunoglobulin Fc effector domains. In these proteins the fused moiety acquires antibody associated properties such as effector functions or improved pharmokinetics. An example is the tumor necrosis factor (TNF) receptor IgG fusion protein, which binds to TNF (a mediator of inflammation) and neutralizes its activity. In fact, this molecule has been demonstrated to be efficacious for the treatment of rheumatoid arthritis (19,20).

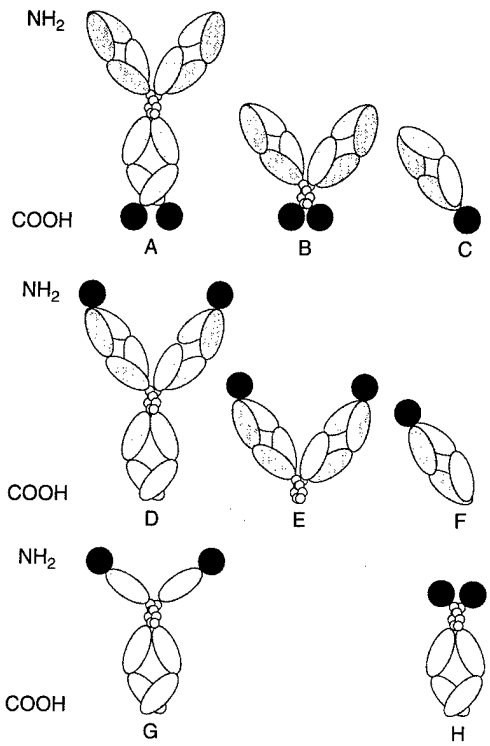

Figure 3. Schematic diagram of Ab fusion proteins. Panels A-C represent different immunoligands in which the ligand was fused at the C-terminus after the C_H3 domain (A), immediately after hinge (B), or after the C_H1 domain (C). Panels D-F represent immunoligands in which the ligand has been joined to the N-terminus of the full-length heavy chain (D) or truncated heavy chain (E and F). Panels G and H represent two immunoadhesins with the adhesin joined to the N-terminus of the C_H1 domain (G) or immediately before the hinge (H).

IN VITRO ANTIBODY PRODUCTION BY PHAGE LIBRARIES

Expression of immunoglobulin genes in filamentous bacteriophage (f1, M13 and fd), viruses that infect bacteria, has been used to obtain Abs with the defined binding specificities (2,5,21). In this strategy, the heavy and light V-gene obtained from spleen of immunized mouse or from peripheral blood of a naive or immunized human donor, are expressed as Fabs or single-chain Fvs (scFv) on the phage surface. Multiple rounds of selection with solid-phase Ag allow isolation of rare phage with variable regions that bind Ag. The selected variable regions will generally have affinities similar to monoclonal Abs and can be expressed as Ab fragments in bacteria (*Escherichia coli.*) or used to produce complete Abs and expressed in mammalian hosts. In addition, specific variable regions can be mutagenized and phage selected that express variable regions with increased affinity.

EXPRESSION SYSTEMS

Abs can be produced in bacteria, yeast, plants, baculovirus and mammalian cells with each expression system having advantages and limitations (2). Complete functional Abs have been most successfully expressed in mammalian cells, as these cells possess the mechanisms required for correct immunoglobulin assembly, posttranslational modification, and secretion. Posttranslational modifications can influence the biological properties and effector functions; important considerations especially when the Ab is to be used for therapy. Other expression system such as bacteria and yeast have been used most effectively to produce Ab fragments such as scFv because the expression of complete functional Abs in those systems presents problems of glycosylation, disulfide bond formation, and assembly.

CONCLUSION

Rapid progress has been made in producing genetically engineered Abs. The ability to express foreign DNA in a variety of host cells has made it possible to produce chimeric, humanized, and human Abs in quantities sufficiently large for many applications including clinical therapy. The available experience suggests that Ab based therapies can be successfully developed for use in clinical situations where no alternative effective therapy is available. However, continued progress in the development of Ab-based therapies will require extensive research to further define the mechanism of Ab action and how to optimally use these novel proteins with unique functional properties.

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ADDITIONAL READING

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integrity as the murine parent. However, $F(ab')_2$ of the humanized Mabs were cleared more rapidly in comparison to the murine $F(ab')_2$ fragments. Size exclusion HPLC of plasma showed the murine $F(ab')_2$ maintained their structural integrity, while the humanized fragments were quickly catabolized. The humanized IgGs were re-engineered to substitute the human IgG_1 hinge sequences with that of the murine IgG_1 orIgG_{2a}, and these newly engineered IgGs maintained stability *in vivo*, but again the $F(ab')_2$ dissociated and were rapidly cleared. Thus, the instability was not a function of the hinge region. Whether these humanized antibody fragments would have similar *in vivo* properties in humans is unknown, but we are continuing to explore possible explanations for this behavior in mice, which might lead to engineering improvements. (Supported in part by a DOE grant ER62028.)

#1824 POTENTIAL CLINICAL APPLICATION OF A NEW MONOCLONAL ANTIBODY 6G7. J Chen, J H Zhou, J Wang-Rodriguez, and E D Ball, *Univ of CA San Diego, La Jolla, CA*

Targeted immunotherapy has made promising progress in the last decade. We developed a new IgG1 murine monoclonal antibody 6G7 (mAb 6G7) and studied its potential clinical application. The mAb 6G7 was raised against the KG-1a cell line and reacted with a cell surface antigen of 220 kDa. We previously showed that mAb 6G7 bound to a subset of CD34+ cells from bone marrow. It reacted with 100% B cells, 40–60% T cell, and 20–40% monocytes. It did not bind to neutrophils, red blood cells, or platelets. Here, we show that mAb 6G7 reacted with 5/5 ALL, 14/16 AML, 1/1 Burkitt's lymphoma, 8/8 CLL, and 7/7 follicular center lymphoma specimens from patients. An immunotoxin was constructed using ricin A chain and mAb 6G7 by chemical conjugation. The growth of KG-1a and Daudi leukemia cells was inhibited by the presence of the immunotoxin in a dose-responsive pattern. The immunotoxin concentration for 50% cell killing ranged from 1–5 nM for Daudi cells and 5–10 nM for KG-1a cells in five experiments. We studied whether the separation of mAb $6G7^+$ cells had any effect on bone marrow progenitor cells by long-term bone marrow culture. The mAb $6G7^+$ and mAb $6G7^-$ cells were separated by cell sorting, and were seeded on irradiated bone marrow stromal layer for long-term culture. The majority of clonogenic cells were in the subset of mAb $6G7^-$ cells. Thus, we conclude that an mAb 6G7 immunotoxin targets the majority of leukemia and lymphoma cells, with minimal effects on hematopoietic progenitor cells. The clinical application of mAb 6G7 deserves further exploration.

#1825 HUMAN BONE MARROW PURGING BY ANTI-CD33 IMMUNOTOXIN. Hatice Duzkale, B Liu, L C Pagliaro, M Korbling, M Rosenblum, A M Tsimberidou, H Kantarjian, B S Andersson, M Beran, A Keyhani, and E J Freireich, *Univ of Texas M D Anderson Cancer Ctr, Houston, TX*

The effects of HuM195-gelonin (H-G) on stem cells (SC) prior to a clinical purging trial was tested. H-G consists of anti-CD33 monoclonal antibody conjugated to gelonin. SC which do not express CD33 antigen were collected from normal donors by apheresis. Clonogenic assays were used to assess cell survival. SC alone and a mixture of SC and HL60 (9:1 ratio, respectively) were incubated with H-G at 1 and 10 nM concentrations for 24 hours. SC alone and the mixture were plated directly after treating with immunotoxin. The treated cells also went through freezing and thawing (F-T) each for 24 hours followed by a 24 hour incubation. Untreated and treated SC were grown in Methylcellulose (MC) supplemented with essential growth factors (GFs) .HL60 cells do not require GFs for clonogenic growth. The mixtures were therefore grown in MC alone, such that the only colonies that found are derived from HL60.The results are: We conclude that HuM195-gelonin in concentrations of 1-10 nM is toxic to leukemic blasts but has little effect on bone marrow stem cells which do not express CD33. For acute myelogenous leukemia blasts which express CD33 this immunotoxin could be useful as a purging reagent for autologous transplantation.

Median Clonogenic Cell Recovery Rate (%)							
	C (MC)	C (MC+GFs)	1nM	10nM	F - T	1nM/FT	10nM/FT
SC	0	100	95	107	64	52	59
Mix	100	- - -	75	62.4	64	6	2

#1826 NEUROBLASTOMA CELL LINES VARY IN THEIR SENSITIVITY TO NEUTROPHIL ANTIBODY DEPENDENT CELLULAR CYTOTOXICITY (ADCC) WITH A HUMANIZED ANTI-GD2/GM-CSF IMMUNOCYTOKINE (HU14.18/GM-CSF). Mark M Podberezin, L. S Metelitsa, S. Gillies, M. Super, N. Keshelava, C. P Reynolds, and R. C Seeger, *Childrens Hosp Los Angeles, Los Angeles, CA, Lexigen Pharmaceuticals Corp, Lexington, MA, and USC Sch of Medicine, Los Angeles, CA*

Hu14.18/GM-CSF was tested in neutrophil (PMN) ADCC against 18 neuroblastoma (NB) cell lines to determine if there is cross-resistance between neutrophil ADCC and chemotherapeutic agents. ADCC was evaluated by a calcein-AM micro-cytotoxicity assay (effector:target ratio, 50:1; hu14.18/GM-CSF, 5μg/ml; 4 hr assay) using digital imaging microscopy (DIMSCAN). Results were expressed as a tumor cell viability index. GD2 expression by cell lines was measured by flow cytometry. Sensitivity of 16 of the lines to cisplatin, carboplatin, melphalan, doxorubicin, and etoposide was evaluated using flourescein diacetate/eosin Y and DIMSCAN. Two neuroblastoma cell lines did not express GD2, while 16 others expressed GD2 (92% to 98% positive cells). As expected, the GD2-negative cell lines were not sensitive to PMN ADCC. The GD2- positive cell lines varied in their sensitivity to PMN ADCC (tumor cell viability index, 28% to 100% for 16 and 28% to 58% for 7 cell lines), but this did not correlate with the level of GD2 antigen expression. The sensitivity to chemotherapy of 14 GD2-positive cell lines varied with 3, 11, 10, 9, and 10 being sensitive to clinically achievable doses of cisplatin, carboplatin, melphalan, doxorubicin, and etoposide respectively. There was no cross-resistance between ADCC and these agents. This study demonstrates that most chemotherapy resistant cell lines are sensitive to PMN ADCC but that there is heterogeneity in sensitivity to ADCC that is independent of sensitivity to chemotherapy.

#1827 THE POSSIBILITY OF UTILIZING HUMANIZED ANTI-PTHRP ANTIBODY AS AN ANTI-HHM/CACHEXIA AGENT. Etsuro Onuma, H. Saito, Y. Azuma, N. Shimizu, T. Tsunenari, K Sato, E. Ogata, *Chugai Pharmaceutical, Shizuoka, Japan; Cancer Institute Hospital, Tokoyo, Japan*

Humoral hypercalcemia of malignancy, HHM, and cachexia are the most frequently occurring paraneoplastic syndromes, and are considered to be serious obstacles to the maintenance of the quality of life of cancer patients. HHM is mediated by tumor derived parathyroid hormone related protein, PTHrP. HHM patients generally develop cachexia, but the exact pathogenesis has not been elucidated. We constructed a humanized anti-PTHrP antibody in order to create a therapeutic agent for the treatment of HHM/cachexia patients. We have investigated the roles of PTHrP in HHM/cachexia using the antibody and PTHrP produced human lung carcinoma LC-6 JCK implanted HHM/cachexia model. The anti-body was administered (in single and multiple doses) intravenously to the HHM model. The antibody showed a normalization of the blood calcium level, rapidly and continuously through the suppression of both bone resorption and renal reabsorption of calcium stimulated by PTHrP. A single dose (3mg/kg) in the HHM model maintained normal blood calcium levels for 10 to 14 days. Moreover, improvements in cachectic symptoms such as the body weight loss, food consumption, water intake and behavior were observed. These results suggest that the antibody should become one of the most effective and beneficial agents for patients with HHM/cachexia.

#1828 *IN VIVO* ANTI-TUMOR ACTIVITY OF AN ANTI-HER-2/NEU-IL-12 ANTIBODY FUSION PROTEIN. Lisan S Peng, Manuel L Penichet, and Sherie L Morrison, *UCLA, Los Angeles, CA*

Interleukin-12 (IL-12) is a heterodimeric cytokine with many actions on innate and cellular immunity that may have anti-tumor and anti-metastatic effects: however, systemic administration of IL-12 can be toxic. Tumor-specific antibodies can be used to selectively target a residual/metastatic nodule and deliver the cytokine to the site of the tumor, ideally limiting toxicity. HER-2/neu over-expression in human breast and ovarian cancers is associated with poor prognosis. Humanized anti-HER-2/neu has been shown to be an effective therapeutic agent in clinical trials and demonstrates that metastatic breast disease can be effectively targeted through the HER-2/neu antigen. We previously reported the construction of an anti-HER-2/neu-IL-12 antibody fusion protein (mscIL-12.her2.IgG3) that retains binding to the HER-2/neu antigen and IL-12 bioactivity. We now report that this fusion protein demonstrates dose-dependent anti-tumor activity. Murine colon adenocarcinoma CT26 cells transfected with the HER-2/neu antigen (CT26/Her2) were injected subcutaneously into BALB/c mice. Treatment with mscIL-12.her2.IgG3 on days 6-10 after tumor implantation was able to arrest tumor growth. Administration of mscIL-12.her2.IgG3 in an experimental CT26/Her2 lung metastasis model also demonstrated anti-tumor activity. When the CT26/Her2 cells were injected subcutaneously into RAG2 -/- mice the anti-tumor effect of mscIL-12.her2.IgG3 was diminished, suggesting that the anti-tumor activity may be mediated in part by T cells. Further work is being carried out to determine the mechanism of the mscIL-12.her2.IgG3 anti-tumor activity.

#1829 B-CELL KILLING BY MONOSPECIFIC AND BISPECIFIC αCD20 ANTIBODIES. Sebo Withoff, Bart Jan Kroesen, and Lou F M H de Leij, *Univ Hosp Groningen, Groningen, Netherlands*

Recently exhilarating results have been obtained in the clinic with the αCD20 monoclonal antibody Rituximab. This antibody is used for treatment of B-cell non-Hodgkin lymphoma patients. It has been suggested that apoptosis induction is an important mechanism involved in Rituximab-mediated cell kill. We are investigating how αCD20 antibodies induce apoptosis in B-cells. Using the αCD20 monoclonal antibody B-ly1 (and gam-crosslinking F(ab')2 fragments) we were able to induce apoptosis in B-cells at antibody-concentrations of 10μg/ml or higher as was determined using the Annexin-V assay. These results confirm published data of others (although other antibodies and other target cell lines were used). Experiments in which caspase-activation is measured in JY and RAMOS cells after B-ly1-induced apoptosis are ongoing. Recently, we have developed an αCD20xαCD3-bispecific antibody (provisionally called BIS20x3) by hybrid hybridoma (quadroma) technology (BIS20x3 was derived from the B-ly1 hybridoma). BIS20x3 could be purified partially from quadroma supernatant by proteinA-FPLC (pH gradient) as was confirmed by FACS and Western blotting. To demonstrate T-cell retargeting by BIS20x3 we have adapted a non-radioactive (calcein-AM-based) CTL-assay for our specific needs. As effectors PBL-derived T-cells were used and JY cells were used as targets. After addition of bsAb 50% cytotoxicity could be demonstrated at an effector-target cell ratio of 5 (ET5). At higher ET-ratio's (ET10 and ET20) respectively 80% and 90% cytotoxicity was

human IL-2 by combining the VH and VL portions of a murine MoAb (520C9) specific for human HER-2 (Li J. et al, Preparation and characterization of a recombinant humanized single-chain Fv antibody/human interleukin-2 fusion protein directed against the HER-2/neu (c-erb B2) proto-oncogene product, p185. Tumor Targeting 4:105-114,1999). The fusion protein is stably expressed in 293 cells and retains the immunostimulatory effects of IL-2 as shown by cell proliferation and cytotoxicity assays. In addition to IL-2 activity, the fusion protein retains full binding activity to p185. Advantages of this fusion protein include improved tissue penetration, increased clearance rate of free antibody and lower immunogenicity. Our preliminary in vivo findings indicate that this fusion protein significantly decreases the growth rate of HER-2 over-expressing tumors in a syngeneic mouse model. Additional studies with the fusion protein are being performed in immuno-suppressed mice carrying human HER-2 positive tumors. Both the tumor growth inhibitory effect and the vasoactive property of the fusion protein will be reported. The latter could cause specific vascular leakage at the tumor site. Such studies will help gain further insight into clinical applications of the fusion protein.

#1565 T Cell and Antibody-Based Immunotherapy of B Cell Lymphoma with Chimeric Fusion Proteins. Kuan- Der Lee, Yan- Chung Shih, Mi- Hua Tao, Mei- Lien Cheng, Hsiung- Kwan Liu, Yi- Ping Lee, Hsin- Wei Chen, Jy- Ping Tsai, Jacqueline Whang- Peng, and Chou- Chik Ting. *IBMS, Academia Sinica, Taipei, Taiwan, and National Health Research Institutes, Taipei, Taiwan.*

Malignant lymphomas are the most common neoplasm of patients between the ages of 20 and 40. With the increasing incidence of AIDS, the number of lymphomas has sharply increased. Indolent lymphomas grow slowly and to date, the survival of these patients is not altered by any chemotherapy, while aggressive lymphomas have an extremely poor prognosis and need high dose combination chemotherapy and usually the hematopoietic stem cell transplantation. Thus, efforts have been made to treat this tumor with immunotherapy. The 38C13 B-cell lymphoma has been studied extensively in the animal model. It has been shown that this tumor could be treated successfully with αId/IL-2 fusion protein but the treatment had to be initiated at a very early stage of tumor growth. We found that the combined use of αCD3-induced killer cells, CD3-AK, with αCD3 antibody could protect mice from tumor growth and the therapeutic effect was augmented by IL-2. However, the systemic toxicity of IL-2 *in vivo* posed a problem. An αCD3/IL-2 fusion protein was thus generated by genetic engineering so that αCD3 could function as a *bi-specific* antibody i.e. as a targeting molecule for B lymphoma cells through the binding of Fc receptor on B lymphoma cells to Fc, and the binding to CD3-AK cells through CD3. In addition, αCD3 is also an activation molecule for T effector cells and the co-conjugated IL-2 would increase T cell survival and avoid systemic toxicity. We have shown that this fusion protein retained the biological activities of both αCD3 and IL-2 in that it could (1) induce T cell activation such as proliferation, induction of CD3-AK, and cytokine production; (2) augment the killing activity of both CD3-AK and LAK cells; (3) maintain T cell growth whereas αCD3 alone failed to do so. It is anticipated that αCD3/IL-2 fusion protein should work better than αCD3 or IL-2 alone or in combination and the *in vivo* therapeutic effect for B lymphoma in combined use with CD3-AK cells will be tested.

#1566 Recombinant Antibody-(IL-2) and Antibody-(GM-CSF) Fusion Proteins for the Treatment of Human HER2/*Neu*expressing Tumors. Jay S. Dela Cruz, K. Ryan Trinh, Seung-Uon Shin, Sherie L. Morrison, and Manuel L. Penichet. *University of California, Los Angeles (UCLA), Los Angeles, CA.*

*HER2/neu*proto-oncogene, also known as *c-erbB-2*,shows markedly increased expression in a subset of human breast, colon, lung and ovarian cancers and this overexpression has been associated with poor prognosis. The elevated levels of the HER2/*neu*protein in malignancies and the extracellular accessibility of this molecule make it an excellent tumor associated antigen (TAA) for tumor specific therapeutic agents. In fact, treatment of patients with advanced breast cancer using the anti-HER2/*neu*antibody trastuzumab (Herceptin, Genentech, San Francisco, CA), directed at the extracellular domain of HER2/*neu*,results in an objective response in a subset of patients with tumors overexpressing HER2/*neu*.To expand the clinical potential of this approach, we have produced two anti-HER2/*neu*antibody fusion proteins with the variable region of trastuzumab and either interleukin-2 (IL-2) or granulocyte/macrophage colony-stimulating factor (GM-CSF) fused at the carboxyl terminus of the Fc fragment of human IgG3. The anti-HER2/*neu*IgG3-(IL-2) and anti-HER2/*neu*IgG3-(GM-CSF) fusion proteins expressed in myeloma cells were correctly assembled and secreted and retained intact antibody and cytokine related activity. Most important, treatment of immunocompetent mice with these antibody fusion proteins resulted in a significant retardation in growth of subcutaneous CT26-HER2/*neu*tumors under conditions in which the anti-HER2/*neu*IgG3 failed to confer protection. We also found that fusing IL-2 or GM-CSF to human IgG3 results in a significant enhancement of the mouse anti-human antibody (MAHA) response. Our results suggest that anti-HER2/*neu*IgG3-(IL-2) and anti-HER2/*neu*IgG3-(GM-CSF) fusion proteins will be useful in the treatment of human HER2/*neu*expressing tumors.

#1567 Immunotherapy of Transgenic Mice with Combinations of Anti-Carcinoembryonic Antigen-II-2 and GM-CSF Fusion Proteins. Xiaochuan C. Xu, Qiong Shi, and Jim F. Primus. *Vanderbilt-Ingram Cancer Center, Nashville, TN.*

The purpose of this study was to examine the therapeutic effects of combination treatment with two antibody-cytokine fusion proteins. IL-2 and GM-CSF were selected because of their ability to stimulate killer lymphocytes and antigen-presenting cells , respectively. Single gene-encoded fusion proteins were prepared that utilized carcinoembryonic antigen (CEA) as the targeting tumor antigen. The fusion proteins were produced as dimeric molecules that had a molecular size predicted by the gene sequences. As determined by surface plasmon resonance, the CEA binding activity (KaffM-1) of the parental antibody, IL-2 fusion protein, and GM-CSF fusion protein was 10.6 x 109, 3.8 x 109, and 0.86 x 109, respectively. When assayed on cytokine-dependent cell lines, the activity of the IL-2 fusion protein was similar to the recombinant cytokine whereas the GM-CSF fusion antibody activity was decreased. Despite the lower antigen binding activity of the GM-CSF fusion protein and a decreased intravascular residence time for both fusion proteins, they selectively localized to CEA-expressing syngeneic MC-38 tumor cells growing in CEA transgenic mice. Therapy with either fusion antibody alone initiated one day after tumor implantation inhibited the growth of CEA-expressing MC-38 tumors when compared to solvent control ($p < 0.05$). Although tumor cures were not obtained with either single or combined treatments, tumor growth was further suppressed and survival times were increased in mice receiving both fusion proteins ($p < 0.01$). The growth of MC-38 tumors expressing an irrelevant CEA-related antigen was not inhibited following fusion protein treatment. These results demonstrate that the antitumor activity of the fusion proteins was dependent upon their tumor targeting properties. Furthermore, the therapeutic efficacy of cytokine fusion proteins may be improved by administering them in suitable combinations.

EXPERIMENTAL/MOLECULAR THERAPEUTICS 17: Antifolates

#1568 Classical, Conformationally Restricted, Linear, Tricyclic 5-Deaza Analogue or Methotrexate as an Antifolate. A. Gangjee, Y. Zeng, J. J. McGuire, and R.L. Kisliuk. *Duquesne University, Pittsburgh, PA, Roswell Park Cancer Institute, Buffalo, NY, and Tufts University School of Medicine, Boston, MA.*

4-(Pyrimido[4,5-*b*][1,6]naphthyridin-7-yl)-methyl-benzoyl-*L*-glutamic acid (1), a novel classical conformationally restricted, linear, tricyclic analogue of methotrexate (MTX: 2), was synthesized via a ten step total synthesis starting from 4 -piperidone, to determine the effects of conformational restriction around the C6-C9 (τ_1)and C9-N10 (τ_2) bonds in MX. Compound 1 was a micromolar to submicromolar (IC_{50}) inhibitor of recombinant human (rh), *P carinii, T. gondii* and *E. coli* dihydrofolate reductase DHFR). Though compound 1 is 240-fold less potent than MTX as an inhibitior of rhDHFR, it its only 60 -fold less potent than MTX as an inhibitor of CCRF-CEM growth in continuous (120 hr) exposure. A subline expressing decreased folylpolyglutamate synthetase (FPGS) activity is 3-fold cross -resistant in continuous exposure, while a DHFR-overexpressing subline and a subline with reduced MTX transport are both >14-fold cross-resistant. The data are consistent with some need for polyglutamylation, transport via the reduced folate carrier, and targeting DHFR. Leucovorin at 10^{-7} M fully protects, consistent with an antifolate mechanism of action. Compound 1 is a 12 fold poorer human FPGS substrate than is aminopterin, primarily because of a high K_m (27 μM). At least one step in the mechanism of action of MTX may thus be sensitive to the conformational restriction of τ_1 and τ_2. Supported in part by AI41743 (AG) , and CA43500, CA16056 (JJM) and CA10914 (RLK)

#1569 Rate of Thymidylate Synthase (TS) Inhibition by CB300638 in the Human A431-FBP Cell Transfected with the α-Isoform of the Folate Receptor (α-FR). Davinder S. Theti, Vassilios Bavetsias, David D. Gibbs, Lorraine A. Skelton, and Ann L. Jackman. *Institute of Cancer Research, Surrey, UK.*

The α-FR is a transmembrane protein functioning in the transport of folates for which it has high affinities (Kd = 0.1-1nM). α-FR expression is generally very low in normal tissues, but is highly overexpressed in some tumours (e.g. 90% of ovarian carcinomas). Thus the α-FR is an attractive target for the selective delivery of chemotherapeutic agents into tumours. Clinically available antifolate TS inhibitors such as raltitrexed and ZD9331 primarily use the ubiquitously expressed reduced-folate carrier (RFC) for cellular uptake. TS inhibitors have now been designed with low and high affinities for the RFC (Km's >100μM) and α-FR respectively (similar to folic acid). One of these is the cyclopentaquinazoline-based compound, 6S-CB300638, which is a potent TS inhibitor (Kiapp = 0.5nM). CB300638 is ~300-fold more potent (IC_{50}=0.003μM) against A431-FBP cells (transfected with the human α-FR) compared to the neo-transfected A431 cells (IC_{50}=1μM). Cells were grown in medium with 20nM LV as the folate source. The rate of TS inhibition (measured by the rate of ^3H_2O release from 5-^{3}H-dUrd) in A431 and A431-FBP cells was used to indirectly monitor uptake of TS inhibitors into the cytosol. ZD9331 (0.8μM; 10x continuous exposure IC_{50}) inhibited TS in A431 cells at 1h (97% inhibition) with recovery after placement in drug-free medium (DFM) for 4h. This is consistent with RFC-mediated uptake. Similarly, 3μM CB300638 gave 80% inhibition at 1h with slow recovery in DFM (60% and 10% inhibition at 1h and 4h respectively). At 0.03μM CB300638, TS inhibition was only seen in the A431-FBP cells but at relatively late time points 0%, ~50%,

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Appendix 11

that $VEGF_{121}$ specifically binds to the flk-1/KDR receptor on vascular endothelial cells and is capable of specific intracellular delivery of the rGel toxin. We designed the novel $GrB/VEGF_{121}$ fusion construct which is a vascular targeting fusion protein composed of a non-heparin binding isoform of VEGF and pro-apoptotic pathway enzyme. Human GrB gene with signal peptide was originally cloned from Hut-78 cells. GrB was attached to $VEGF_{121}$ via a short, flexible tether (G_4S). The fused gene was then cloned into pET32a (+) vector. The 40 kDa $GrB/VEGF_{121}$ fusion protein was expressed in E. coli and purified by Ni-NTA metal affinity chromatography. The His-tag was removed after digestion with recombinant enterokinase leaving the first residue Ile of mature GrB exposed. Western blotting using either anti-$VEGF_{121}$ or anti-granzyme B antibody confirmed incorporation of both $VEGF_{121}$ and GrB proteins into the construct. BAADT Assay indicated that the construct maintained GrB enzymatic activity similar to that of native GrB. $GrB/VEGF_{121}$ fusion protein demonstrated specific binding (ELISA) to PAE/flk-1 cells overexpressing the flk-1/KDR receptor but showed no binding to PAE/flt-1 cells overexpressing the flt-1 receptor. *In vitro* cytotoxicity of $GrB/VEGF_{121}$ against PAE/flk-1 vs PAE/flt-1 cells showed that the IC_{50} was ~10 nM against PAE/flk-1 cells, however, there were no cytotoxic effects on PAE/flt-1 cells. Treatment of PAE/flk-1 cells with the fusion construct resulted in cleavage of caspase-8, caspase-3, PARP and DFF45 within 4 hrs after administration but this was not observed on PAE/flt-1 cells after $GrB/VEGF_{121}$ treatment. In conclusion, we demonstrated that delivery of human pro-apoptotic pathway enzyme GrB to tumor neovascular endothelial cells or to tumor cells may have significant therapeutic potential for cancer treatment and represents a potential new class of targeted therapeutic agents with a defined mechanism of action. Research conducted, in part, by the Clayton Foundation for Research.

#5031 Monoclonal antibody against prostate stem cell antigen (PSCA) inhibits prostate cancer growth and metastasis through a direct effect on tumor cells. Robert E. Reiter, Zhennan Gu, Joyce Yamashiro, and Evelyn Kono. *UCLA School of Medicine, Los Angeles, CA.*

PSCA is a 123 amino acid cell surface glycoprotein expressed in normal prostate and bladder. We have previously shown that PSCA protein is overexpressed in 40% of local and as many as 100% of metastatic prostate cancers. Increasing expression of PSCA correlates with advanced tumor stage and grade. Anti-PSCA mAb was shown by us to be able to prevent prostate tumor establishment, growth and metastasis in a xenograft model of prostate cancer. The present study was to determine whether the *in vivo* activity of PSCA moncolonal antibody was mediated by the immune system (Fc dependent) or by a direct effect of antibody on tumor cells. The ultimate goals of this study are to enhance the efficacy of antibody therapy directed against PSCA and to understand the role of PSCA in tumor progression. The *in vivo* effects of whole PSCA antibody 1G8 (IgG1) were compared with its F(ab)2 fragment in order to determine if the anti-tumor effects of mAb 1G8 were dependent on the presence of the Fc domain. Equimolar amounts of whole 1G8(200 micrograms) and F(ab)2 were administered intraperitoneally to nude mice inoculated subcutaneously with LAPC-9 prostate cancer cells. Antibody was administered beginning on the day of tumor inoculation (tumor take experiment) or when tumor became palpable (tumor inhibition experiment). The effect of mAb 1G8 on the *in vitro* growth of prostate cancer cells was also investigated. Briefly, LNCaP or LNCaP cells stably transfected with PSCA (LNCaP-PSCA) were treated with 1G8 or control antibody. Cells were assayed for growth at various timepoints after exposure to antibody. Intact mAb 1G8 was able to inhibit tumor take and tumor growth significantly compared to PBS. The inhibitory effect of the F(ab)2 fragment of mAb 1G8 was equal to or slightly less (80%) than whole mAb 1G8. F(ab)2 prevented tumor establishment and prolonged survival of mice (in the tumor take experiment) simlar to whole antibody. Likewise, F(ab)2 was able to slow growth of established tumor similar to whole antibody. Interestingly, in a number of animals, we saw tumor regression after exposure to F(ab)2. F(ab)2 was internalized into prostate cancer cells more rapidly than whole antibody, which may be relevant to its efficacy. In the *in vitro* assays, mAb 1G8 inhibited proliferation of LNCaP-PSCA by 80% at 72-96 hours, whereas it had no effect on the growth of LNCaP cells that did not express PSCA. Antibody treated cells had striking morphologic changes at 24 hours after antibody treatment, which are in the process of being characterized. The studies suggest that PSCA mAb 1G8 inhibits prostate cancer growth *in vitro* and *in vivo* primarily through a direct, Fc-independent mechanism and suggest that PSCA may play an important role in prostate cancer progression. These results are being confirmed further in FcR knockout mice. The mechanism(s) by which PSCA antibodies exert their direct effect are also being investigated.

#5032 Influence of cumulative dose (CD) of trastuzumab administered with taxanes vs pre-treatment CD of anthracyclin on left ventricular ejection fraction (LVEF) in metastatic breast cancer (MBC) patients. Benedicte Aunoble, Anne-Chantal Braud, Jean-Michel Bons, Xavier Durando, Lionel Moreau, Isabelle Van Praagh, Jean Maublant, Herve Cure, Patrice Viens, and Philippe Chollet. *Centre Jean Perrin, Clermont-Ferrand, France, Centre Paoli Calmettes, Marseille, France, and Centre Jean Perrin, INSERM U484, Clermont-Ferrand, France.*

Trastuzumab treatment in Her-2 positive patients is given with taxanes, because of frequent cardiac toxicity if administered in association with anthracyclin (Baselga J., E.J.C., 2001). Previous anthracyclin CD is proposed to explain the frequent LVEF decrease observed during trastuzumab therapy (Fornier, Sem. Oncol., 2000). Could also the trastuzumab CD play a role in LVEF decrease? From July 1999, 33 MBC patients were treated with weekly trastuzumab (4 mg/kg loading dose then 2 mg/kg), associated with weekly paclitaxel (18/33) or 3-weekly docetaxel (15/33). Median age patient was 55 years [28-70]. Median pre-treatment anthracyclin CD and trastuzumab CD were respectively 411 mg/m^2 [0-665] and 36 mg/kg [4-86]. Treatment discontinuation was due to disease progression (10/33), LVEF decrease (6/33) (estimated by scintigraphy or echography), end of treatment (5/33), bad general condition (1/33); 11/33 patients are still treated. According to the NCI Common Toxicity Criteria, nearly half of all patients (15/33) presented with cardiac toxicity. LVEF decrease included 9 patients with Grade I and 6 patients with Grade II. Three out of nine Grade I cardiac toxicity (decrease of 15 and twice 18 % from LVEF baseline value) were seen in patients without previous anthracyclin. We studied the relationship between 1) the anthracyclin CD (in equivalent dose of adriamycin, with a factor 0.7 for epirubicin and 7 for mitoxantrone) or 2) the trastuzumab CD and the longitudinal LVEF variation. Using a linear test, LVEF decrease was shown to be significantly correlated with trastuzumab CD ($p < 10\text{-}6$) but not with anthracyclin CD ($p = 0.21$). Then the cumulated dose of trastuzumab administered with taxanes could be more relevant than pre-treatment anthracyclin CD for LVEF decrease in MBC patients. In our opinion, a cardiac monitoring should be done when trastuzumab is administered in combination with taxane (independently of previous anthracyclin treatment).

#5033 Anti-HER2/*neu* antibody fusion proteins: Effective enhancers of extracellular domain HER2/*neu* protein vaccination. Jay S. Dela Cruz, Ernesto M. Ramirez, Suk Ying Lau, Carla De Giovanni, Guido Forni, Sherie L. Morrison, and Manuel L. Penichet. *University of California at Los Angeles, Los Angeles, CA, and University of Bolgna, Bologna, Italy.*

HER2/*neu* is overexpressed in some human breast, ovarian, prostate and lung cancers and is associated with poor prognosis. DNA and peptide based vaccines targeting HER2/*neu* have elicited significant protection against HER2/*neu* expressing cancers in animal models. However, vaccines using the complete extracellular domain of HER2/*neu* (ECD-HER2) have not shown the same efficacy. We have developed several anti-human HER2/*neu* antibody fusion proteins (Ab-FPs) containing the immunostimulatory cytokines: IL-2, IL-12 or GM-CSF. These Ab-FPs retain both cytokine activity and the ability to bind ECD-HER2. To determine if these Ab-FPs can act as immunoenhancers for ECD-HER2 vaccination, mice were vaccinated with human ECD-HER2, ECD-HER2 with anti-HER2/*neu* Ab, or ECD-HER2 with each anti-HER2/*neu* Ab-FP. After a booster, mice were challenged with a syngeneic carcinoma expressing the rat HER2/*neu* (TUBO). There was a significant retardation of tumor growth rate and long-term survivors in mice vaccinated with ECD-HER2 plus all three Ab-FPs as compared to the control groups (PBS, ECD-HER2 or ECD-HER2 plus anti-HER2/*neu* Ab). Anti-(ECD-HER2) humoral immune response was detected in all vaccinated groups, with ECD-HER2 plus Ab-(GM-CSF) and ECD-HER2 plus Ab-(IL-2) vaccinated mice showing the highest titers. These two groups had increased level of anti-(ECD-HER2) IgG1 and IgG2a Abs, as compared to the control groups, suggesting both T_H2 and T_H1 immune responses were elicited; while Ab-(IL-12) vaccinated mice showed increased IgG2a Abs but not IgG1 Abs, suggesting a T_H1 immune responses was elicited. Immune sera showed significant *in vitro* anti-proliferative activity against SK-BR-3 (a human breast cancer with overexpressed HER2/*neu*), with the level of inhibition correlated with the level of anti-(ECD-HER2) Ab. When incubated with soluble ECD-HER2, splenocytes from mice vaccinated with ECD-HER2 plus Ab-(GM-CSF) demonstrated significant stimulation and IFN-γ secretion as compared with the other groups. Our results suggest that both humoral and cell-mediated responses may contribute to the observed anti-tumor activity. Further studies are now in progress to extend these results. Our studies suggest that anti-HER2/*neu* Ab-FPs may be effective prophylactic and therapeutic agents against HER2/*neu* expressing tumors in patients. Importantly, patients with high levels of circulating shed ECD-HER2, who are not currently eligible for anti-HER2/*neu* Ab based treatments, may benefit.

#5034 Development of less immunogenic antibodies derived from an anti-carcinoma antibody reactive against carcinoembryonic antigen. Roberto De Pascalis, Makoto Iwahashi, Midori Tamura, Eduardo Padlan, Noreen Gonzales, Ameurfina Santos, Mariateresa Giuliano, Peter Schuck, Jeffrey Schlom, and Syed Kashmiri. *LTIB, CCR, NCI, NIH, Bethesda, MD, LMB, NIDDK, NIH, Bethesda, MD, and MIR, DBEPS, ORS, NIH, Bethesda, MD.*

Murine monoclonal antibody (MAb) COL-1 reacts with carcinoembryonic antigen (CEA), which is expressed on a wide range of human carcinomas. In preclinical studies in animals and clinical trials in patients, murine COL-1 showed excellent tumor localization. To obviate the problem of immunogenicity of the murine antibody in patients, genes encoding the heavy and light chains of MAb COL-1 were cDNA cloned and a mouse-human chimeric COL-1 was developed. Subsequently, a humanized COL-1 (HuCOL-1) was generated by grafting the MAb COL-1 complementarity determining regions (CDRs) onto the light- and the heavy-chain frameworks of two human MAbs, while retaining those murine framework residues that may be critical for antigen binding. To minimize anti-idiotypic responses of patients to HuCOL-1, a variant of HuCOL-1 was generated, by grafting onto the human frameworks, only the 'abbreviated' CDRs that contain the specificity determining residues (SDRs) and are essential for ligand contact. The recombinant antibodies were purified and characterized. In competition radioim-

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PATENT APPLICATION

Antibody Fusion Proteins: Effective Adjuvants of Protein Vaccination

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